THE INVESTIGATION OF SIRTUIN ACTIVITIES ON SHORT-CHAINED MONO-ACYLATED HISTONE H3 TETRAMERS AND NUCLEOSOMES

A Dissertation

by

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ABSTRACT

Lysine acylation serves as an epigenetic marker for myriad cellular processes, such as signaling, differentiation, DNA repair, angiogenesis, and the like. Sirtuin 1 (SIRT1) and Sirtuin 2 (SIRT2) are NAD^+-dependent histone deacylases that operate as posttranslational regulators of lysine acylation states. Despite being a topic of increasing interest, little effort has been made to completely understand the overall character of these two sirtuin enzymes. Most of the studies have also been on the peptide level and not on actual sirtuin substrates (i.e. histone H3/H4 tetramers and nucleosomes). Thus the demand for systematic studies of enzymatic activities on native substrates for SIRT1 and SIRT2 is currently needed.

In this dissertation, we discuss the ability for SIRT1 and SIRT2 to deacylate various short-chain acylations—acetylation, crotonylation, and butyrylation—from lysine residues on the tetramer and nucleosome substrates. In addition, we explore the difference in selectivity between using peptide and nucleosome substrates with sirtuins. We find that SIRT1 and SIRT2 demonstrate relatively no substrate specificity across the screened histone H3 sites, contrary to previous expectations.

The implication of the overall non-specificity of SIRT1 and SIRT2 on the tetramer and nucleosome substrates suggests that these sirtuin enzymes have an adaptive nature, harnessing an ability to respond to various cellular situations, rather than an enzyme specifically designed for a particular task or function.

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Contributors

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The compounds used in Chapter IV for the ncAA incorporation into histone H3 was synthesized and provided by Dr. Yadagiri Kurra of the Department of Chemistry.

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NOMENCLATURE

Acetyl-CoA	Acetyl coenzyme A
AcK	N^{ε}-Acetyl-L-lysine
ACSS2	Acyl-coenzyme A synthetase short-chain family member 2
ATP	Adenosine triphosphate
APC/C	Anaphase-promoting complex
BAX	bcl-2-like protein 4
BuK	N^{ε}-Butyryl-L-lysine
Butyryl-CoA	Butyryl coenzyme A
CAF-1	Chromatin assembly factor-1
CBP	CREB-binding protein
CrK	N^{ε}-Crotonyl-L-lysine
Crotonyl-CoA	Crotonyl coenzyme A
ELISA	Enzyme-linked immunosorbent assay
EPL	Expressed protein ligation
GNAT	Gcn5-related *N*-acetyltransferases
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
Ku70	Lupus Ku autoantigen protein p70
MYST	MOZ, Ybf2, Sas2, Tip60 family
NAD^+	Nicotinamide adenine dinucleotide

ncAAs	Noncanonical amino acids
NCL	Native chemical ligation
Ni-NTA	Nickel nitrilotriacetic acid
P300	E1A binding protein p300
PTMs	Posttranslational modifications
QM/MM	Quantum mechanics/molecular mechanics
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sir2	Yeast sirtuin 2
SIRT	Sirtuin
SPPS	Solid phase peptide synthesis

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CHAPTER I

INTRODUCTION

Histones

In eukaryotes, 147 bp of chromosomal DNA is wrapped and organized 1.7 times around the histone octamer to form the nucleosome. Each histone octamer contains a pair of histone H2A, H2B, H3, and H4 proteins **(Figure I-1)**. Each nucleosomal unit is linked together to form a beads-on-a-string structure called chromatin.[1] The four histone proteins have a similar helix-turn-helix structure and a long N-terminal tail that lacks any defined secondary structure. All four histone proteins are the target of numerous, strictly regulated posttranslational modifications, such as acetylation, methylation, crotonylation, butyrylation, sumoylation, ubiquitination, and myriad other acylations. These modifications have the ability to alter nucleosomal stability and chromatin compaction, as well as, the ability to act as effectors of downstream cellular functions.[2-6]

Over the past decades, much progress has been made in the identification of new posttranslational modifications on histones as well as the enzymes involved in their modification; however, histones were not always viewed with such scrutiny as they are today. After its discovery in 1884 by Albrecht Kossel, histones remained largely dismissed by the scientific community as a static source for DNA packaging. It was not until the late 20th century when a study found that transcription would not initiate on the nucleosome; however, if transcription were initiated before reaching the nucleosomal core, the process of transcription would proceed normally while displacing the histones

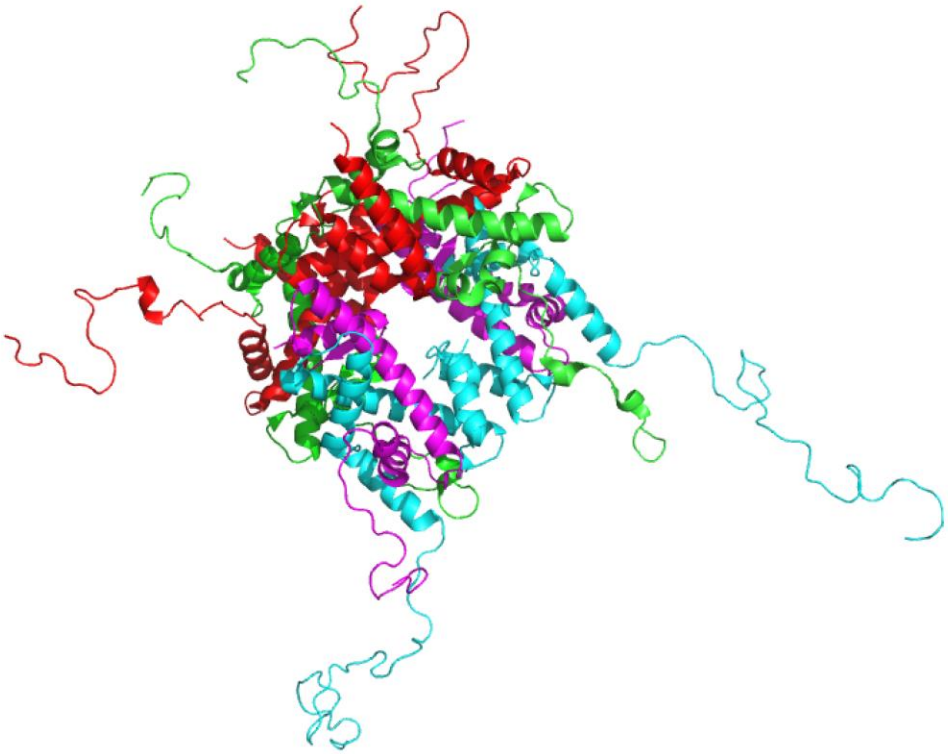

Figure I-1. Crystal structure of the histone octamer. Histone H2A is denoted in green, H2B in red, H3 in cyan, and H4 in magenta. PDB: 1KX5

from the DNA during the course of transcription.[7] A companion study later demonstrated a similar repressing effect on the nucleosome *in vivo*.[8] From there, the nucleosome began to find acceptance in the scientific community as a general gene repressor, rather than as an inert DNA packing complex. And eventually, with the discovery of histone-modifying enzymes, such as Gcn5 and Sir2 in yeast, the link between the nucleosome and posttranslational modifications was drawn.

In the following decades, scientists have discovered that the nucleosome is the core to many diverse and complex cellular functions in the cell, including an active part in cell repair, gene transcription and repression, cell signaling, chromatin remodeling, DNA replication, and the like.[2-6]

Posttranslational Modifications

With some exceptions, the twenty canonical amino acids are the building blocks of all eukaryotes; however, it is well known that to meet the complexity and rigors of the eukaryotic cell, these amino acids need to undergo chemical modifications termed posttranslational modifications (PTMs).[9] These modifications grant proteins the ability to be involved in basic cellular and metabolic functions as well as cellular localization. These PTMs range from short to long chain acylations, three different stages of methylation, small molecule acylation, and more.[10-12] It is well known that these PTMs are reversible and tightly regulated in cellular systems to maintain cell growth and health.[6, 13-16] Lysine posttranslational modifications are the most prominent PTM in living cells, where the side-chain amino group can undergo a variety of acylations **(Figure I-2)**. Herein, we will discuss the importance and presence of the various lysine acylations in more detail.

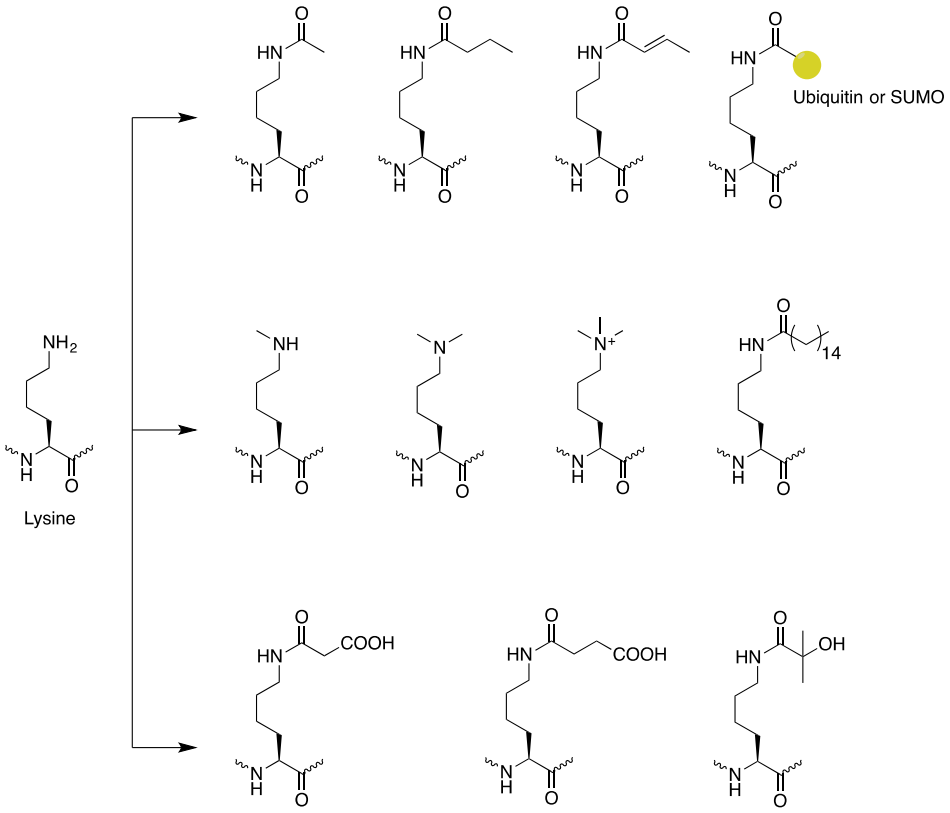

Figure I-2. Protein lysine acylation.

Protein Lysine Acetylation

Protein lysine acetylation is one of the most prominent posttranslational modifications found *in vivo*. Acetylation occurs in cell via the catalytic transfer of the acetyl group from an acetyl coenzyme A (acetyl-CoA) molecule onto the side-chain amino group of lysine residues via histone acetyltransferases (HATs). One of the major roles of protein acetylation is its role in the translocation of newly synthesized histones from the cytoplasm to the nucleus via specific acetylation patterns. During the S phase of the cell cycle, newly synthesized histone H3 and H4 are produced while older histone H3/H4 tetramers are marked for degradation.[17-20] These newly synthesized histone H3 and H4 proteins are then acetylated at various specific sites (depending on species) that act as a signal for them to be taken for assembly into nucleosome. One such example is the function of human chromatin assembly factor-1 (CAF-1), which can freely pass between the cytoplasm and nucleus. CAF-1 detects and binds to newly synthesized histone H3 and H4 proteins, likely in the tetrameric form, in the cytoplasm and deposits the histones onto DNA in the nucleus.[21] An interesting feature of CAF-1 is its ability to recognize the difference between new histone H3 and H4 from its mature counterpart.[22] This is likely due to differences in specific acetylation patterns on the histone proteins. Once the nucleosome has been formed, these acetylation patterns are then deacetylated by histone deacetylases (HDACs) and modified with new posttranslational modification patterns to utilize them for various cellular processes.

Another critical role of protein acetylation is its role in regulating chromatin compaction. Chromatin exists in two states: euchromatin and heterochromatin **(Figure I-**

3). The term euchromatin is defined as the state in which chromatin is loosely packed whereas heterochromatin is characterized by the tightened chromatin state. The conversion of lysine to acetyllysine via a histone acetyltransferase is known to alter the electrostatic interactions between the lysine amino side-chain and DNA. In a study by the Ottesen group, they were able to demonstrate the DNA loosening affect of acetylated histone H3 lysine 56 (H3K56ac) on the nucleosome structure.[23, 24] Histone H3K56ac is a known site on the core region of the nucleosome that very closely interacts with the DNA unlike most of the other lysine sites on histone H3, which are located on the very flexible and unstructured *N*-terminal tail. However, the acetyl group is not solely the source of changing euchromatin to heterochromatin and vice versa. The acetylation marker acts as a signal (in conjunction with other specific PTMs) for nucleosome remodeling complexes to bind to the nucleosome region. The acetyllysine contributes by repelling the DNA enough to create space for the remodeling complexes to dock onto that region, where an unmodified lysine cannot accommodate that demand.

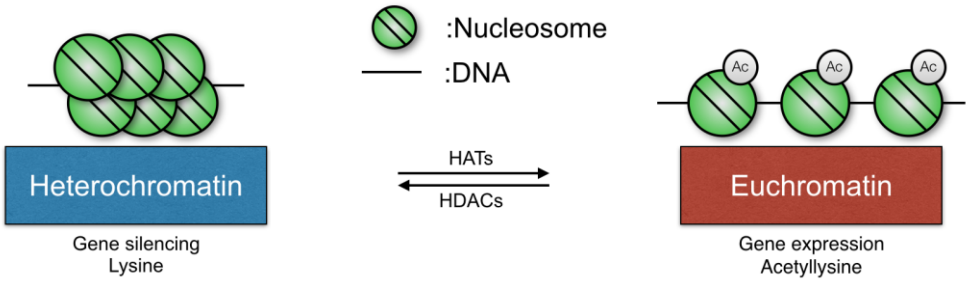

Figure 1-3. Chromatin in two states: heterochromatin and euchromatin.

Histone H4 is unique in its contribution to chromatin compaction via a single acetylation site histone H4K16ac.[25] When considering the structure of chromatin, the *N*-terminal tail of histone H4 rests in the acidic region of a neighboring histone H2A/H2B dimer via hydrogen bonds and salt bridges, which consists of six histone H2A and two histone H2B residues.[26-28] Acetylation at histone H4K16 negates the interactions in this region and forces chromatin to begin to loosen slightly. In subsequent studies, deletion of the histone H4 *N*-terminal tail (residues 1-19) demonstrated a similar chromatin state, suggesting that histone H4K16 is vital for chromatin compaction, whereas H4K16ac is a hallmark for chromatin loosening.[25] In addition, *in vitro* reconstitution of nucleosome arrays with histone H4K16ac demonstrated no difference from the nucleosome array with the *N*-terminal tail of histone H4 deleted.

The transfer of the acetyl moiety from acetyl-CoA to a lysine residue is catalyzed by histone acetyltransferases (HATs), and the removal of the acetyl moiety from acetyllysine is catalyzed by histone deacetylases (HDACs). Histone acetyltransferases are generally categorized into two families—the Gcn5-related *N*-acetyltransferase (GNAT) family and the MOZ, Ybf2, Sas2, Tip60 (MYST) family. Other acetyltransferases that do not fit in these two families due to either larger or mutated variants of the HAT domain, such as p300, CBP, and Rtt109 are placed in an unlabeled category.[29] These categorizations are mainly based on sequence homology and shared structural features. One major difference between the GNAT and MYST family is the catalytic residues involved in the acetyltransferase mechanism (**Figure I-4 & I-5)**.

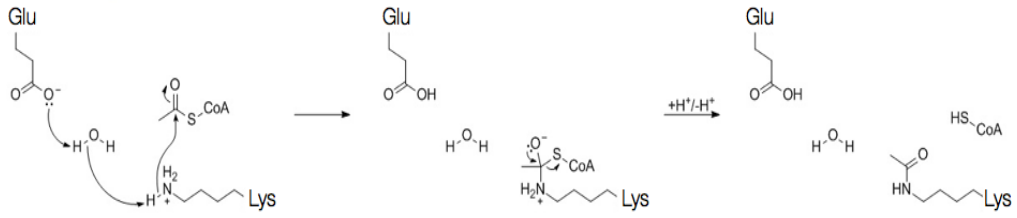

Figure I-4. GNAT family acetyltransferase mechanism.

The family of Gcn5-like acetyltransferases has a conserved glutamate residue in the active site that serves to active a nearby water molecule that then activates the lysine substrate for the nucleophilic attack on an acetyl-CoA molecule.[30] Following the collapse of the tetrahedral intermediate, one free CoA molecule and acetylated lysine residue is afforded. The catalytic core of the MYST family has a conserved glutamate residue as well; however, this family of enzymes also has a conserved Zn^{2+}-binding site and a cysteine rich region, which are involved in the catalytic mechanism. The MYST family's acetyltransferase mechanism involves a ping-pong mechanism where acetyl-CoA first binds to the active site pocket where is undergoes nucleophilic by a cysteine residue, creating a transient acetylated cysteine residue and one free CoA molecule, which unbinds from the active site. The lysine substrate then binds to the pocket where it undergoes activation by the nearby glutamate residue and attacks the carbonyl of the acetylated cysteine. Collapse of the tetrahedral intermediate yields an acetylated lysine product, which then leaves the active site last.[31]

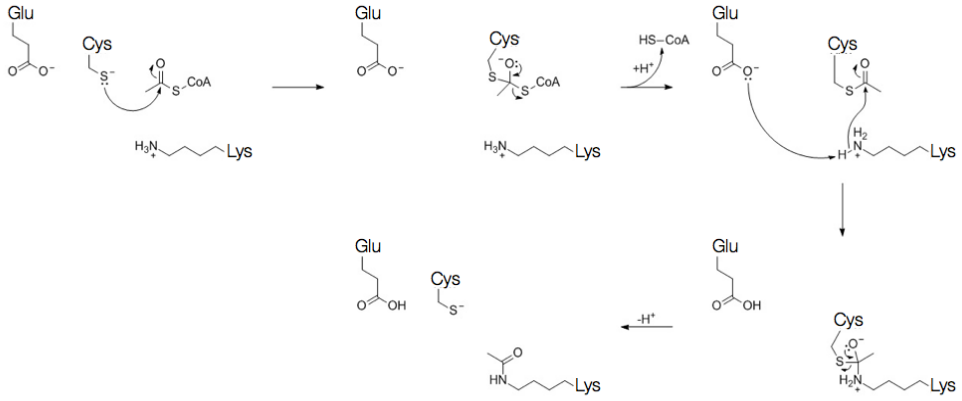

Figure I-5. MYST family acetyltransferase mechanism.

Conversely, the enzymes that remove the acetyl moiety from acetyllysine are dubbed histone deacetylases. Histone deacetylases are divided into four classes, where Class I, II, and IV HDACs are Zn^{2+}-dependent enzymes and Class III HDACs (also called sirtuins) are NAD^{+}-dependent. The sirtuin mechanism of action for the deacetylation of acetylated lysines will be reviewed in Chapter 2. For Class I, II, and IV HDACs, the zinc ion in the active site interacts with two aspartates, one histidine, and two nearby water molecules **(Figure I-6)**.[32, 33] Upon the entrance of acetyllysine, a nearby histidine residue activates one of the two water molecules bonded to the Zn^{2+} which then attacks the carbonyl group, forming a tetrahedral intermediate. Following the collapse of the tetrahedral intermediate, the acetyllysine is deacetylated and lysine is released.

Another interesting structural feature of this mechanism is the presence of a conserved distal tyrosine residue that exhibits two distinct conformations termed out and in.[33] In the absence of any acetyllysine substrate, the tyrosine residue adopts an outward-facing formation, where no contacts with the active site are made. However, upon the entrance of acetyllysine into the active site, the enzyme adopts a conformation change, which then orients the tyrosine inward, creating a hydrogen bonding interaction with the oxygen of the acetyl moiety.

In this dissertation, we study the site specificity of SIRT1 and SIRT2 on histone H3 acetylated nucleosomes.

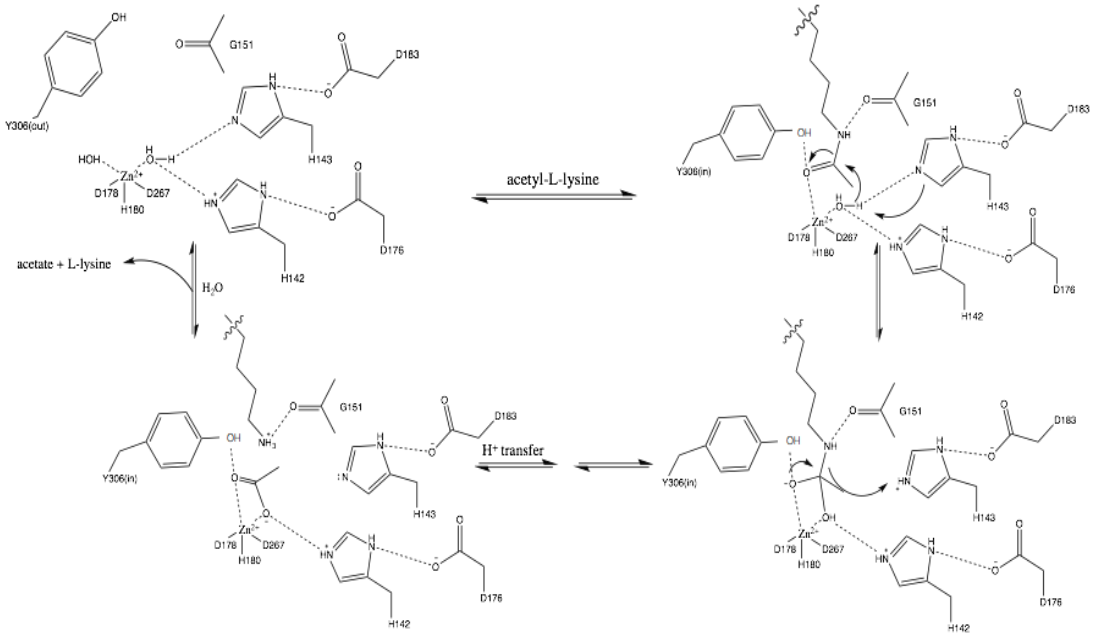

Figure I-6. Catalytic mechanism for the deacetylation of acetyllysine by HDAC8.

Protein Lysine Crotonylation & Butyrylation

Protein lysine crotonylation and butyrylation are two new acylations found on the histone protein. In 2007, a discovery by the Yingming Zhao group found two novel posttranslational modifications on histones: propionylation and butyrylation.[34] Since its discovery, butyrylation has been found to be catalyzed by histone acetyltransferase p300. Another study by the Khochbin group found that histone H4K8bu is a transcriptionally activating marker. In addition, histone H4K8bu competes with acetylation at the same site in order to achieve the same overarching goal of gene transcription; however, the two compete posttranslational marks recruit different downstream binders.[35] Later in 2011, the Yingming Zhao group once again discovered another posttranslational modification on histones—crotonylation.[36] This particular PTM was intriguing in that the mark seemed to propagate in gene promoter regions. Later studies demonstrated that crotonylation is associated with enhancers and active transcription start sites. Similar to butyrylation, histone acetyltransferases are able to catalyze the transfer of the crotonyl moiety from crotonyl-CoA onto histones.[37]

Theories on the regulation of crotonylation and butyrylation outside of enzymatic interactions suggest that intracellular concentrations of crotonyl-CoA and butyryl-CoA may be involved in the regulation of its specific PTM marker. A study conducted by the Sabari group, hypothesized that by downregulating acyl-CoA synthetase (ACSS2) in cells, there would be a decrease in the global concentration of histone crotonylation.[37] Their theory was found to be correct by the decrease in histone H3K18cr, suggesting that

ACSS2 has a role in producing crotonyl-CoA in cells. In a follow up study, Sabari et al. found that the typical concentration of crotonyl-CoA in cells is generally lower than that of acetyl-CoA; however, by purposely downregulating the production of acetyl-CoA, the concentration of crotonyl-CoA increases.[37, 38] This discovery suggested that acetyl-CoA and crotonyl-CoA might have competing roles in the modification of histone lysine sites.

However beyond these preliminary studies, much of crotonylation and butyrylation remains a mystery. Are butyryl-CoA and crotonyl-CoA possible substrates of histone acetyltransferases? What enzymes catalyze the decrotonylation and debutyrylation of the modified histone proteins? In this dissertation, we probe the deacylation ability of these two PTMs with SIRT1 and SIRT2 *in vitro*.

Histone Crosstalk

As an effect of posttranslational modifications, histone proteins undergo a phenomenon called crosstalk—the activation or inhibition of a specific site's modification as a direct result of another site's modification. Histone crosstalk can occur in a manner of ways. Cross regulation *in trans* occurs when the modification target is not on the original histone substrate whereas *in cis* cross regulation occurs on the same histone (**Figure I-7)**. Histone cross regulation can occur cross-nucleosome as well, modifying either contiguous nucleosomes or non-contiguous nucleosomes.

An example of crosstalk is when histone H3S10 phosphorylation inhibits histone H3K9 acetylation and promotes histone H3K14 acetylation.[39-41] Another type of crosstalk involves the promotion of one exclusive mark over another. Histone H3K9 can be either acetylated or methylated exclusively on a global level depending on the transcriptional state.[42, 43] In other words, histone H3K9ac at one site can promote the acetylation of other nearby histone H3K9 sites to promote active transcription. Conversely, histone H3K9me can promote the methylation of other histone H3K9 sites to repress transcription.

One final common example of histone crosstalk is the promotion of acetylation on the histone H4 N-terminal tail. Histone H4 has five critical lysine residues at histone H4K5, H4K8, H4K12, H4K16, and H4K20. Acetylation at any of these five sites will promote the acetylation of the other four, favoring an active transcriptional state.[44]

Each of these modifications, both singular and combinatorial, closely regulate specific cellular functions within the cell. Additionally, certain crosstalk patterns can be

exclusive to one species. For example, in *S. pombe*, the acetylation of histone H3K14 and methylation of histone H3K9 are antagonizing, whereas in human cells, histone H3K9me and H3K14ac can coexist on the same histone H3 tail.[42, 45]

In a study probing the potential of histone acetyltransferase Gcn5 bromodomain-mediated crosstalk, Shogren-Knaak and coworkers discovered that bromodomain recognition of histone H4K16ac promoted cooperative histone H3 acetylation at various sites.[46, 47] By assembling arrays 12 nucleosomal units in length with acetylation at histone H4K16, their lab was able to demonstrate preferential acetylation by Gcn5 at histone H3K14ac, followed by acetylation at histone H3K9, H3K18, and H3K23. This analysis was the first *in vitro* crosstalk study.

Previous crosstalk discoveries were observed *in vivo*, where the mechanism of crosstalk spanned across multiple enzymes in a complex; however, being able to isolate and simplify one of the many crosstalk models is the first step toward better understanding the individual pieces of histone crosstalk. Other histone-modifying enzymes, such as TIP60, SUV39H1, and SIRT1, are all involved in some form of crosstalk on the nucleosome, necessitating the need to better understand the individual enzymes as the first step toward fully understanding their involvement in histone crosstalk.[48-52] In this dissertation, we choose to study the site and substrate specificity of SIRT1 and SIRT2.

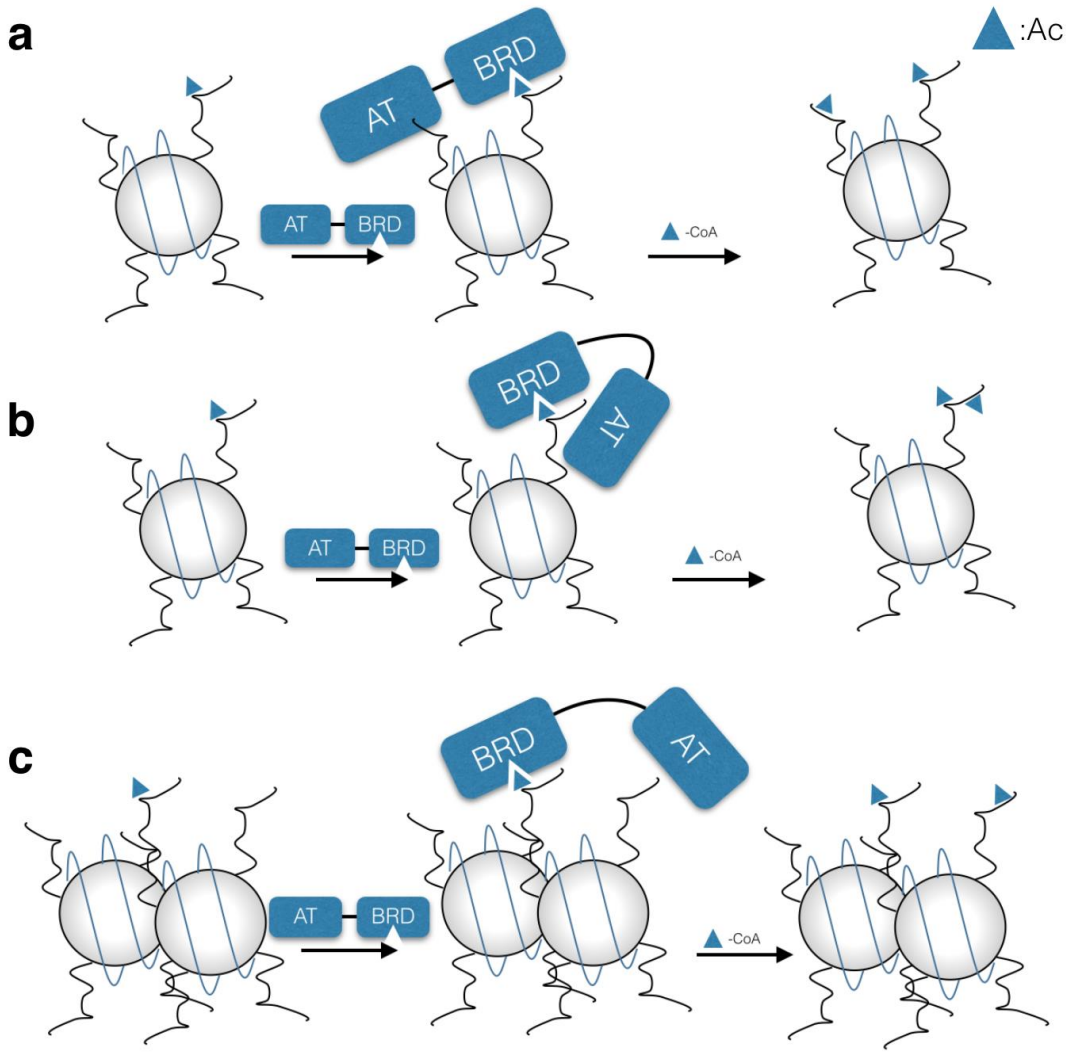

Figure I-7. Histone crosstalk can occur in three ways: (A) *in trans*, (B) *in cis*, (C) and cross-nucleosome.

Synthetic Noncanonical Amino Acid Incorporation

In order to study these histone proteins and their modifications, the need for a method of producing histones with defined posttranslational modifications is necessary. In the past, the most common practice for the study of histones was to extract the histone proteins from varying cell lines; however, because of the dynamic nature of posttranslational modifications in the cell, obtaining nucleosome or even histone protein with an isolated PTM or a specific combination of PTMs is difficult, if not impossible. As a solution, many scientists who wish to study the affect of specific PTMs in an *in vitro* environment looked to solid phase peptide synthesis (SPPS). SPPS is a convenient tool for the synthesis of peptides up to about 50 amino acids in length; however, since most native proteins with PTMs are over 50 amino acids, it is not an ideal solution.[53] Additionally, SPPS is a very expensive technique that is not available ubiquitously.

Native Chemical Ligation

The first major breakthrough and the most traditional method of incorporating noncanonical amino acids (ncAAs) was native chemical ligation (NCL), which involves the reaction between the *N*-terminal cysteine of one peptide with the *C*-terminal thioester of another. The reaction begins with the thiol group on the cysteine exchanging with the thiol group on the thioester. The reaction creates a new thioester linkage which then undergoes an intramolecular rearrangement to produce the final combined peptide (**Figure I-8)**.[54] Since the reaction involves the conjugation of two peptides synthesized by solid phase peptide synthesis, this method allows for the synthesis of proteins up to

100 amino acids in length. However, there are two major limitations: (1) SPPS is an expensive and tedious technique with low yield and (2) the desired protein will have a cysteine residue in its peptide sequence. In native chemical ligation, following the synthesis of desired peptide, the reaction is commonly never 100% efficient, meaning that the product itself must be further purified by column chromatography, most likely high performance liquid chromatography (HPLC), which further decreases the already low yield of the peptide. In most cases this limitation of yield can be sidestepped by scaling down the assay conditions; however, in nucleosome reconstitution, a large amount of protein/peptide is needed for the successful refolding, which makes nucleosome study highly unlikely with NCL. Additionally, since one of the precursor peptides must have a cysteine residue for native chemical ligation to proceed, the product will have a native cysteine in its protein sequence. This case is not ideal when the protein of interest does not have a cysteine in its native protein sequence, making this technique highly protein selective.

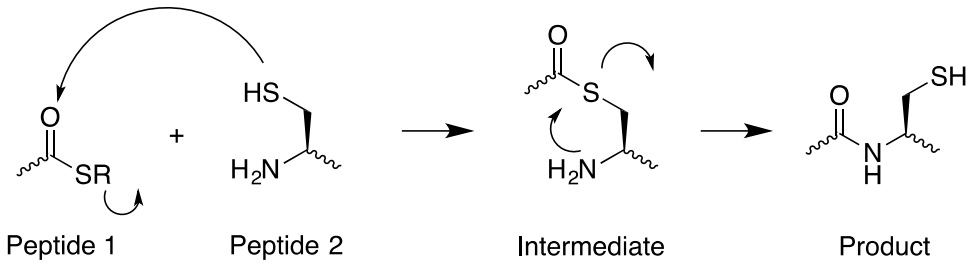

Figure 1-8. Native chemical ligation.

Expressed Protein Ligation

The next step in the chemical synthesis of posttranslationally modified proteins came with the development of expressed protein ligation (EPL).[55] EPL uses a technique termed protein splicing, which is a technique that involves an intramolecular rearrangement to remove an internal sequence and join two remaining sequences via the same chemistry as NCL to produce a protein amide bond.[56, 57] By using recombinant protein technology, a protein sequence of choice is fused to intein on the *C*-terminal followed by an affinity-binding domain, most commonly chitin-binding domain. A peptide with an *N*-terminal cysteine is then synthesized using SPPS. The protein is then bound to a column via its corresponding affinity tag and treated with another thiol bearing nucleophile, which then allows the protein with the *C*-terminal thioester to react with the *N*-terminal cysteine peptide and form the desired product **(Figure I-9)**. This new method removes the 100 amino acid limitation since bacterially expressed proteins are not limited to 50 amino acids. In addition, the cost of such expressed proteins is much lower than SPPS with the added benefit of higher yields. However, many of the limitations of native chemical ligation remain with expressed protein ligation—protein selectivity, possible mutated protein sequence, and the need for extra purification.

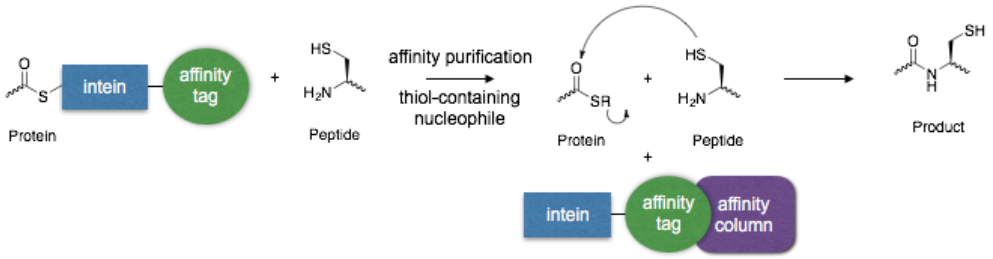

Figure I-9. Expressed protein ligation.

Synthetic Preparation of Histones

Recently, a method popularized by the Ottesen lab for the synthetic preparation of histone H3 with posttranslational modifications employs a modified NCL approach, but uses three peptide fragments instead of two.[58] Histone H3 has various acetylation sites in its 134 amino acid sequence that cannot be synthesized using regular EPL and NCL techniques, such as histone H3K56 and H3K79. In this method, in addition to the synthesis of a C-terminal thioester (Fragment 1) and an *N*-terminal cysteine peptide (Fragment 2), an additional peptide with both a protected *N*-terminal cysteine and *C*-terminal thioester (Fragment 3) is synthesized. Fragments 2 and 3 are then ligated together using conventional NCL chemistry. Following completion, the thiazolidine is deprotected to generate a free terminal cysteine, which is then reacted with Fragment 1 to yield full-length histone H3. Although this approach allows for the study of previously unattainable sites on histone H3, this approach does not solve the previous limitations of NCL—cost and yield—but merely exacerbates the situation; however, this is the only current way to synthesize full-length histone proteins with modifications in the middle of the proteins.

Genetic Noncanonical Amino Acid Incorporation

With the increasing interest in studying posttranslational modifications, a new method for incorporating noncanonical amino acids into proteins using a genetic approach was developed by Peter Schultz and coworkers. The technique was inspired by the discovery of the 21st and 22nd amino acids, selenocysteine and pyrrolysine, respectively.[59, 60] Selenocysteine and pyrrolysine were both particularly fascinating discoveries in that they employed stop codons for their incorporation into proteins—UGA and UAG, respectively.[61, 62] Whereas the normal 20 amino acids have multiple codon recognition combinations, both selenocysteine and pyrrolysine have only one codon recognition site. This is significant in that neither of these two amino acids compete with another amino acid for incorporation. With its aminoacyl-tRNA synthetase and cognate tRNA, the tRNA is charged with the amino acid at the cost of one ATP molecule. The anticodon region of the tRNA then binds to the codon corresponding to the amino acid on the mRNA strand, following which the amino acid is then added onto the growing protein chain (**Figure I-10)**. Schultz and coworkers use this genetic machinery as a model to introduce an orthogonal tRNA/aaRS into cells, meaning the pair only work together and cannot compete with other tRNA/aaRS pairs.[63, 64] Schultz and coworkers eventually developed a system employing a $tRNA_{CUA}^{Tyr}$/tyrosyl-tRNA synthetase pair from the archaea *M. jannaschii* expressed in bacterial *E. coli*.[64] Following suit, the Liu group has taken full advantage of the $tRNA_{CUA}^{Pyl}$/pyrrolysyl-tRNA synthetase pair for the expression of multiple phenylalanine and lysine derivatives.[65-71]

The genetic noncanonical amino acid (ncAA) approach has strengths where previous synthetic ncAA incorporation failed. Bacterial expression is more cost efficient and available to any research group with an interest in chemical biology. In addition, since the proteins are recombinantly expressed, the protein yields are multiple times larger. The need for multiple purification steps is also generally negated with the introduction of affinity tags, such as HisTag, GST Tag, etc.[72-74] Even the ability to express proteins with two different ncAAs has been achieved in the past, albeit with low yield.[75] With the rising demand in studying posttranslational modifications relating to the nucleosome, the need for a method in which histone protein can be expressed quickly and efficiently is needed. The genetic noncanonical amino acid approach provides such a way to meet this demand and is thus used as the primary method of expression for all histone proteins in this dissertation.

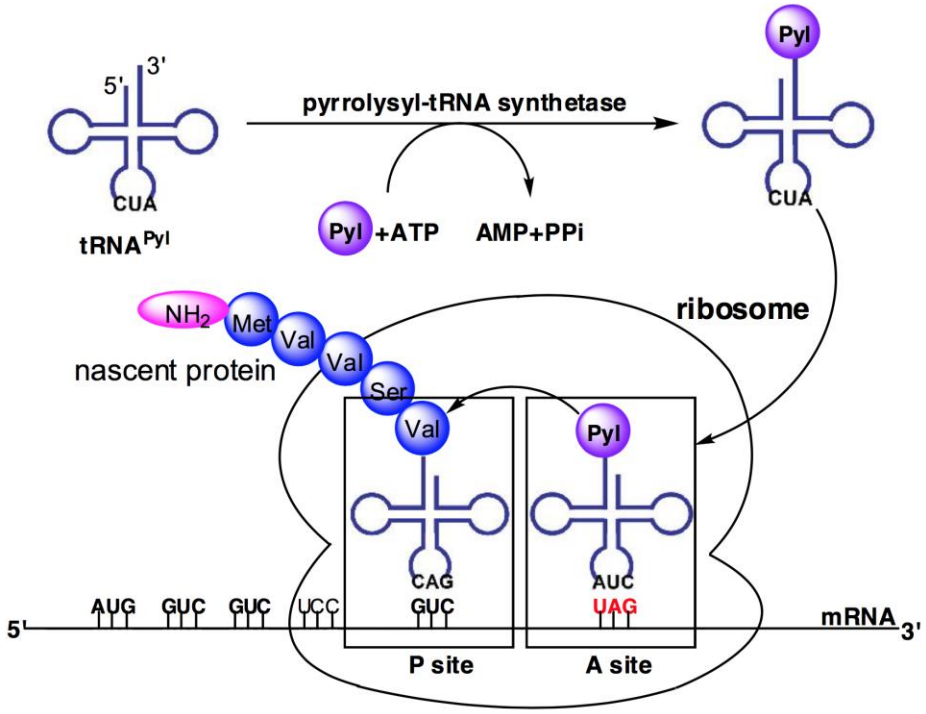

Figure I-10. Genetic ncAA incorporation machinery using a pyrrolysine model.

CHAPTER II

SIRTUIN

Introduction

Sirtuins are Class III histone deacetylases that require NAD^+ as a co-substrate for the catalytic removal of the acetyl moiety from acetyllysine. The first sirtuin enzyme, yeast Sir2, was first discovered in 1979 and was described as a protein required in the silencing of HM loci.[76] Later that year three more proteins were discovered with similar functions and the name silent information regulator (Sir) proteins were assigned to this new group of proteins.[77] It wasn't until 1999 when Kaeberlein and coworkers reported that overexpression of Sir2 had the ability to extend the lifespan of yeast by up to 70%.[78] From this discovery, a significant rise in interest in sirtuins was created.

Later studies found seven enzymes in mammalian cells with strong similarities to yeast Sir2 and termed SIRT1-7.[77] Localization of these sirtuins varies within the cell. Sirtuin 1, 2, and 6 are mainly nuclear enzymes, although SIRT2 is able to shuttle between the cytoplasm and nucleus depending on the cell state. Sirtuin 3-5 are mainly mitochondrial enzymes. Sirtuin 7 is localized in the nucleus as well; however, the enzyme is most commonly found in the nucleolus. The sirtuin family of enzymes have gained much interest in the past couple decades for their ability to not only catalyze the deacetylation of acetyllysine, but also for their ability to act as regulators of health and metabolism.[79-84] Most interestingly, most sirtuins have been confirmed to have the ability to perform a variety of deacylations beyond acetylation, such as crotonylation,

butyrylation, octanoylation, and myristoylation (**Figure II-1b)**.[13] Given the large scope of substrates these mammalian sirtuins can target, the need for more research become more significant in order to fully understand the role and function of sirtuins specifically within the cell. In this dissertation, SIRT1 and SIRT2 reactivity on several acylation sites will be described in detail.

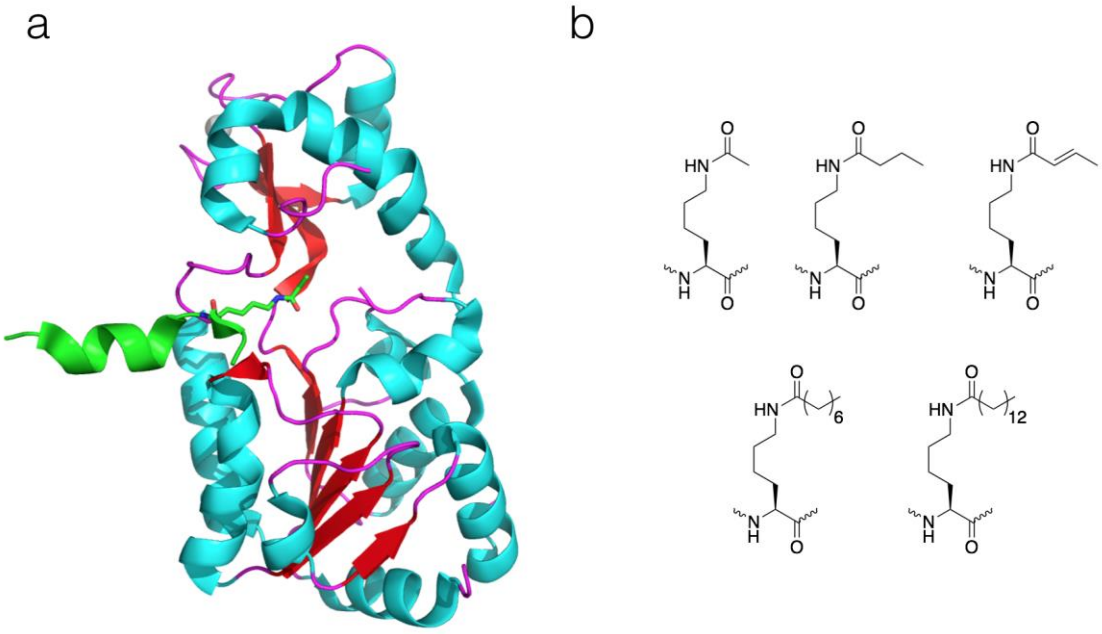

Figure II-1. A) Crystal structure of SIRT2 bound to acetyllysine. PDB: 3PDH B) Five sirtuin substrates.

Mechanism

Structural studies of SIRT2 have shown when the substrate protein and NAD^+ are bound in their respective binding pockets, the oxygen on the acetyl group attacks the nicotinamide at the C1' position of the ribose ring. The nicotinamide structure is then ejected from the catalytic pocket and an alpha-1'-*O*-alkylamidate intermediate is formed. A nearby histidine residue then activates the 2'-OH group on the ribose ring, which attacks the carbonyl of the intermediate and forms a bicyclic intermediate. Hydrolysis of this intermediate releases the deacetylated lysine as well as one *O*-acetyl ADP ribose group (**Figure II-2)**. A high cellular concentration of nicotinamide has been shown to inhibit this mechanism at the formation of the alkylamidate intermediate.[85, 86]

The collapse of the bicyclic intermediate has long been a point of contention. A study by the Zhang lab has shown that a critical water molecule located in a water channel close to the cofactor binding loop forms a hydrogen bond with the 1' oxygen atom of the intermediate.[87] Their mechanism proposed based on *ab initio* quantum mechanics/molecular mechanics (QM/MM) simulations is as follows: First, the bond between the C and O1' is broken, which is stabilized through interactions with the nearby water molecule. Next, nucleophilic attack by water creates a tetrahedral intermediate, which eventually collapses to yield one free lysine and *O*-acetyl ADP ribose group (**Figure II-3)**. A point of interest in this mechanism is that the positioning of the water molecule must be shielded prior to its role in the collapse of the bicyclic intermediate. Otherwise, hydrolysis of the *O*-alkylamidate intermediate would yield a hydrolyzed ADP ribose product. This shielding effect is achieved through a patch of

hydrophobic residues. As the deacetylation mechanism proceeds, the sirtuin enzyme slightly shifts the positioning of this hydrophobic cluster in order to allow or deny access to the cofactor-binding site based on which intermediate is currently present. In addition, this particular structural feature is highly conserved throughout the sirtuin family, implying that the enzymatic design of this mechanism is deliberate.[87, 88]

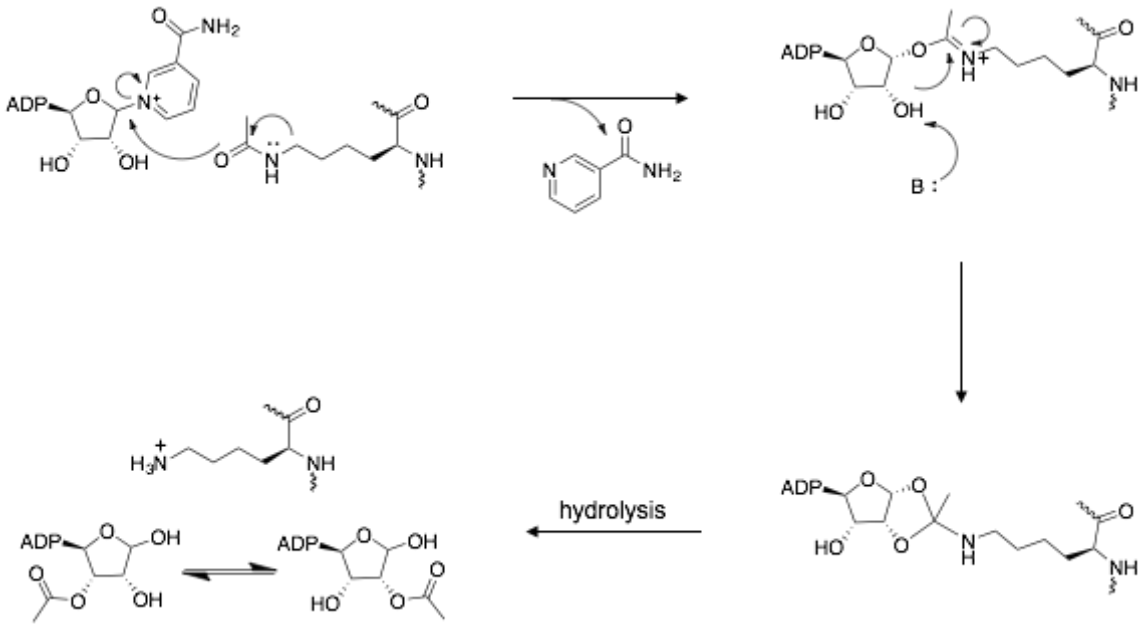

Figure II-2. Sirtuin deacetylation mechanism.

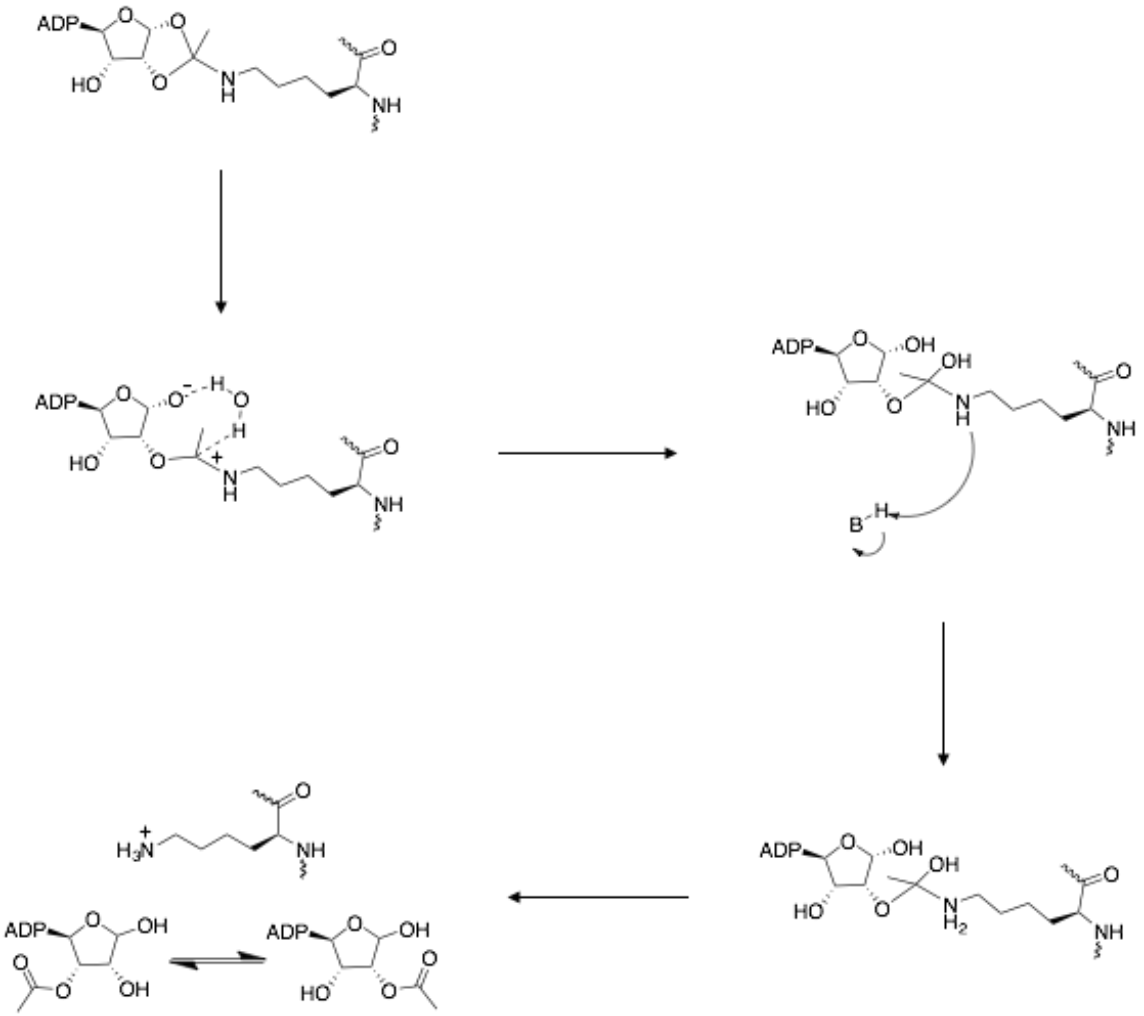

Figure II-3. Proposed mechanism for the collapse of the bicyclic intermediate.

SIRT1 and SIRT2 as Histone Deacylases

At the time of discovery, SIRT1 and SIRT 2 were found to deacetylate histone H3K9ac, H3K14ac, and H4K16ac *in vivo*, similar to its yeast Sir2 counterpart. These modifications listed are confirmed gene activating marks on chromatin by promoting the change from euchromatin to heterochromatin with the help of binding proteins.[88-91] Beyond these initial studies, continued studies of sirtuin enzymes on the nucleosome and even the histone protein ceased due to lack of accessible experimental methods to synthesize or express homogenously posttranslationally modified histone proteins. Preliminary efforts incubated acetylated histone peptides generated by solid phase peptide synthesis with SIRT1 and SIRT2 *in vitro*; however, the deacetylation rates of their acetylated peptides were very low, raising questions as to whether or not they were actual deacetylation targets.[91, 92] Studies have confirmed histone H3K9ac and H4K16ac as SIRT1 and SIRT2 deacetylation sites, and histone H3K14ac as another with slower activity, but a large majority of the histone sites remained non-targets.[89, 93]

In order to better understand the role of SIRT1 and SIRT2 *in* vivo, Oberdoerffer and coworkers discovered in a study that SIRT1- and SIRT2-deficient mice have an increase in DNA damage, which caused downstream irregularities leading to various cancers.[94] In addition, Reinberg and coworkers managed to develop a knockdown SIRT1 cell line, which was characteristic of hyperacetylated levels of histone H3K9 and H4K16, regular SIRT1 targets. Conversely, overexpression of SIRT1 led to the deacetylation of histone H3K9ac and H4K16ac, leading to the eventual downstream pause of gene expression.[91] Another study by Dryden et al. found that SIRT2 has the

ability to control the mitotic exit in the cell cycle. Downregulation of SIRT2 in this study demonstrated a majority of cells being shut down at the G2/M phase of mitosis.[95] Other studies have found that sirtuin has the ability to regulate lifespan in a diet-dependent manner related to calorie restriction and suppress tumorigenesis by regulating APC/C activity.[96] However, details of how SIRT1 and SIRT2 were able to perform these actions on the chemical level were still not understood.

Through the efforts of the Liu group, it was found that SIRT1 and SIRT2 target a wide variety of histone H3 sites with acetyllysine, crotonyllysine, or butyryllysine installed (**Figure II-4 & II-5)**.[97] SIRT1 and SIRT2, which were long known to demonstrate tight substrate specificities on peptide substrates, showed almost no substrate specificity on the nucleosome or tetramer level. This discovery was significant in that the implications of an enzyme with a wide substrate scope in both site specificity as well as PTM-type specificity helped bridge the understanding between SIRT1 and SIRT2's role in regulating cellular health in the cell. It was concluded that SIRT1 and SIRT2 have the ability to regulate and police the presence of specific posttranslational modifications at specific moments in the cell cycle, as a defender of cellular health and longevity.

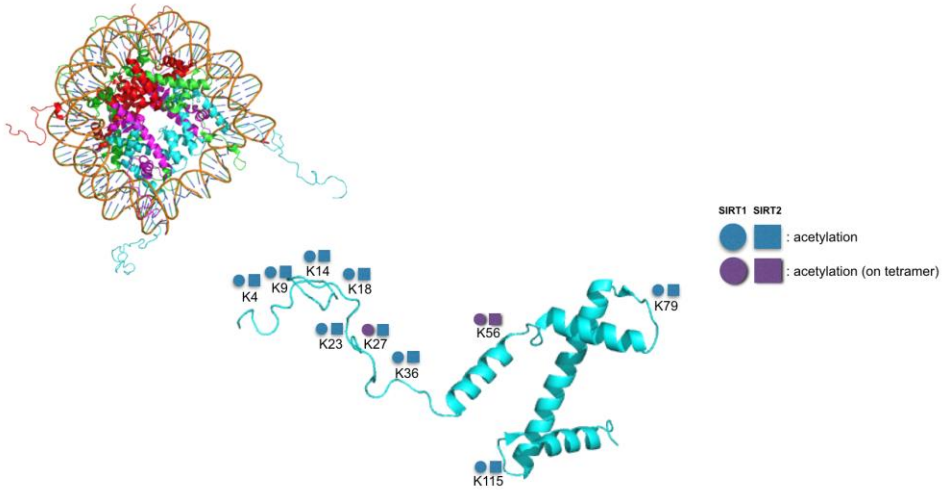

Figure II-4. Deacetylation profile of SIRT1 and SIRT2 on the nucleosome. PDB: 1KX5

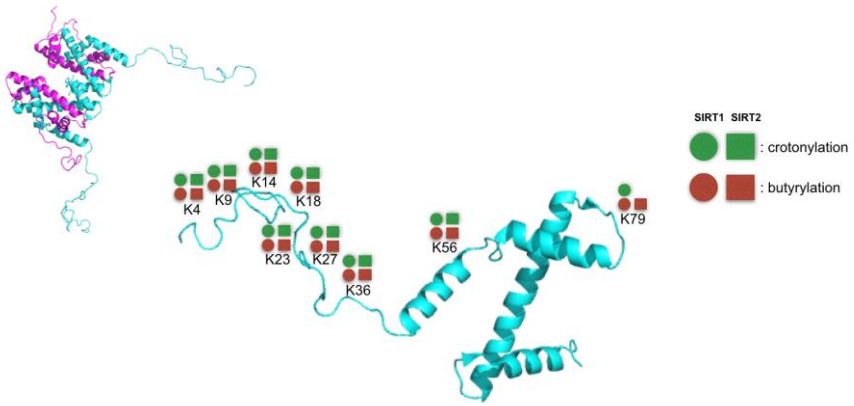

Figure II-5. Decrotonylation and debutyrylation profile of SIRT1 and SIRT2 on the histone H3/H4 tetramer. PDB: 1KX5

SIRT1 and SIRT2 in Transcriptional Roles

Another critical function of SIRT1 and SIRT2 is in its involvement in DNA damage and repair.[88, 98] Usually under moments of oxidative stress in the cell, SIRT1 plays a major role in DNA damage maintenance by recruiting and activating repair proteins. A study by Wang et al. demonstrated that in mice with the SIRT1 gene knocked out, there was an increase in the number of damaged DNA sites on chromatin as well as a noticeable failure in the DNA repair machinery.[98] This study concluded that SIRT1 did in fact play critical roles in the process of DNA repair. Another study demonstrated SIRT1's ability in inhibiting the apoptotic-favored Ku70-BAX disassociation machinery.[99, 100] In response to DNA damage, Ku70 is normally acetylated at multiple sites, breaking its interaction with BAX, also known as bcl-2-like protein 4. BAX then translocates to the mitochondria where it induces cellular apoptosis. However, in the presence of SIRT1, the acetylation sites of Ku70 are removed, maintaining the interaction between Ku70 and BAX, preventing apoptosis and promoting Ku70-mediated DNA damage repair.[99]

SIRT2 demonstrates a similar role in DNA maintenance, but through other means. As mentioned earlier, SIRT2 is most commonly localized in the cytoplasm; however, during cell division, SIRT2 translocates into the nucleus to act as a manager in cell division, regulating several key steps in mitosis.[93, 96, 101] To begin, SIRT2 has the ability to regulate anaphase by promoting APC/C (anaphase-promoting complex) activity. At the beginning of metaphase, APC/C is inhibited; however, by deacetylating the co-activators of APC/C, APC/C is activated, allowing for the complex to mark

proteins for degradation and push the mitotic cycle from metaphase into anaphase.[96] SIRT2 has also been demonstrated to act as a regulator of the G2/M phase. During this phase, SIRT2 functions as a key regulator in chromatin compaction by deacetylating histone H4K16ac and promoting the monomethylation of H4K20. A study done in SIRT2-deficient mice demonstrated an inability to methylate histone H4K20, which resulted in cellular cessation of mitosis at the G2/M phase. In addition, the same study demonstrated that the same SIRT2-deficient mice exhibited characteristic signs pointing to tumorigenesis.[102]

CHAPTER III

DEVELOPMENT OF AN ELISA-BASED RAPID THROUGHPUT ASSAY FOR THE ENZYMATIC PROFILING OF SIRT1 AND SIRT2 ON TEN MONO-ACETYLATED HISTONE H3 NUCLEOSOMES*

Introduction

Lysine acetylation on nucleosomal histones serve as prominent epigenetic marks for gene regulation, which are installed by histone acetyltransferases and removed by histone deacetylases (HDACs).[103] There are four classes of HDACs. Unlike traditional classes I, II, and IV HDACs that are Zn^{2+}-dependent, class III HDAC enzymes are NAD^{+}-dependent called sirtuins.[104] Sirtuin 1 (SIRT1) and Sirtuin 2 (SIRT2) are two sirtuin enzymes that can translocate between the nucleus and the cytoplasm.[105] Recent studies indicate that these two enzymes regulate necessary cellular processes such as metabolism, cellular health, as well as genomic stability.[106-111] Indeed, SIRT2-deficient mice have demonstrated an increase in DNA damage, which acts as the preliminary force for the development of various cancers.[96, 102, 112] This is characteristic of $SIRT1^{-/-}$ mice as well.[94, 98]

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Of their regulatory duties, a prominent function of SIRT1 and SIRT2 is their role in gene silencing and DNA repair, which is achieved via the deacetylation of acetylated histone lysine residues.[113] Previous *in vitro* studies have reported deacetylation of acetylated histone H3 lysine 9 (H3K9ac), H3K14ac, and H4K16ac by SIRT1 and histone H3K9ac by SIRT2, which are confirmed gene activating post-translational marks on histones.[88, 93, 113, 114] However, beyond the initial studies, limited efforts have been made to completely understand the overall character of these two enzymes on the nucleosome. Past studies have perceived SIRT1 and SIRT2 to demonstrate substrate selectivity; however, their spacious substrate-binding sites seem to suggest otherwise.[91, 92, 114] In addition, most of the previous *in vitro* studies have used histone N-terminal tail peptide substrates that may not properly represent their native nucleosomal counterparts.[13, 91, 92] Using peptides as substrates discounts the information related to the contribution of the nucleosome core and other histone tails involved in the enzymatic deacetylation process. Unlike the acetyllysine sites on the histone N-terminal tails that may rearrange in a variety of orientations to interact with SIRT1 and SIRT2, the acetyllysine sites near or in the nucleosome core region such as K36, K56, K79, and K115 of histone H3 have more stringent conformational environments and lack the flexibility of peptides when interacting with SIRT1 and SIRT2. In this regard, using peptides as substrates can lead to potentially misleading results. We aim to resolve these issues related to the current activity studies of SIRT1 and SIRT2 using homologous nucleosome substrates that are site-specifically installed with acetyllysine.

In this work, we implement an integrated method combining our ability to incorporate noncanonical amino acids with an ELISA-based rapid throughput assay approach to survey which lysine sites on histone H3 can be targeted by SIRT1 and SIRT2. Our experimental set up allows for up to sixteen concurrent time-dependent enzymatic assays at once, which permits for the expedient profiling of enzymatic substrate specificity on the nucleosome. In this manner, we were able to characterize the ability of SIRT1 and SIRT2 to deacetylate nucleosomes with mono-acetylated histone H3 over a four-hour time course. In this study, we report that SIRT1 and SIRT2 display relatively little substrate specificity across various histone H3 acetyllysine sites on the nucleosome. In addition, both SIRT1 and SIRT2 display heightened enzymatic activity when incubated with a nucleosome substrate versus its peptide counterpart. This discovery potentially indicates that the nucleosome core and other histone tails have roles in contributing to the binding of and the subsequent deacetylation by SIRT1 and SIRT2.

Experimental Details

Construction of Plasmids

In order to express the various histone proteins for nucleosome assembly, four separate plasmids were constructed using an empty pETDuet-1 plasmid as the backbone: pETDuet-H2A, pETDuet-H2B, pETDuet-H3, and pETDuet-SUMO-TEV-H4. The histone DNA fragments were amplified out of their respective plasmids and ligated into the second MCS of an empty pETDuet vector. A forward primer was specially designed to include the DNA sequence for a HisTag-TEV linker. In this way, each histone sequence was preceded by a HisTag-TEV linkage at the *N*-terminus. BL21(DE3) cells were transformed to hold the expression vectors.

pETDuet-SUMO-TEV-H4 was designed after the pETDuet-H4 construct demonstrated poor yield over multiple expression cultures. The cloning procedure was similar to the construction of the other three histone plasmids. First, two new restriction sites were introduced between the HisTag DNA sequence using QuikChange mutagenesis. SUMO was then cloned into the new region using a forward primer containing the DNA sequence for HisTag. In this manner, a DNA sequence culminating in His-tagged SUMO-TEV-H4 was designed where after TEV cleavage, the native histone H4 protein could be obtained.

Construction of Histone H3 Mutant Plasmids

The aforementioned human histone H3 gene was cloned into a pETDuet-1 plasmid. In following, a site-specific amber mutation was made to the wild-type gene by

Quikchange mutagenesis in order to obtain the desired mutagenic feature (H3K4TAG, H3K9TAG, etc.) BL21(DE3) cells were transformed with the histone H3 mutant and its respective pEVOL construct for expression.

Histone H2A, H2B, H3 Expression and Purification

N-terminal His-tagged wild-type H2A, H2B, and H3 were grown in 2×YT media to OD_{600} 0.40–0.60 and then supplemented with 0.800 mM IPTG to induce expression for 4 hours at 37ºC. Cells were then collected by centrifugation at 4 K for 20 minutes. Following resuspension with a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% NaN_3, and 0.1% Triton X-100, the cells were lysed by sonication at 4ºC. For a general 1 L expression, 40 mL of lysis buffer was used for resuspension. The cell lysate was then centrifuged at 6 K for 20 minutes and the pellet was collected. The pellet was then resuspended in a lysis wash buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.1% NaN_3 and centrifuged at 6 K for 20 minutes. This process was repeated another time under the same conditions. The resulting pellet was then collected an dissolved in a urea buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 6 M urea and incubated at 37ºC for 1 hour. The solution was then cleared by centrifugation at 10 K for 40 minutes.

The supernatant was then collected and subjected to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare). The column was washed with a wash buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 20 mM imidazole, and 6 M urea for at least 10 column volumes. After the column was washed adequately, the protein was eluted

with a second buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 300 mM imidazole, and 6 M urea. Following purification, the proteins were then dialyzed to a temporary storage buffer containing 20 mM Tris-HCl (7.5), 500 mM NaCl, and 6 M urea two times at 4^{o}C. The protein absorbance at Abs_{280} was then obtained and the concentration was calculated using a known extinction coefficient. For long-term storage, the proteins were then dialyzed into water four times at 4^{o}C, lyophilized and stored at -80^{o}C.

Generally, the yields of wild-type histone H2A and H2B were around 10-15 mg/L while histone H3 had a yield of around 25 mg/L. The purity of these three proteins is shown in Figure III-1.

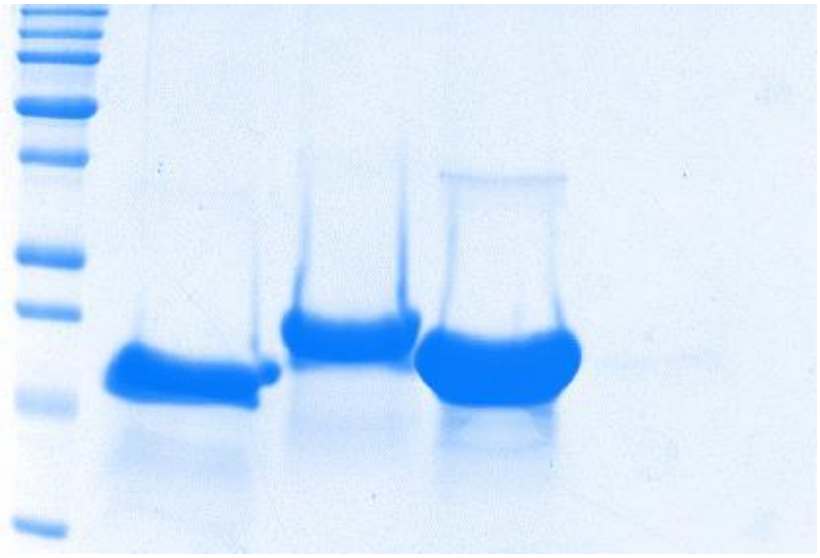

Figure III-1. 15% SDS Page gel depicting the wild-type expression of His-tagged histone H2A, H2B, and H3.

Histone H3 Mutant Expression and Purification

Mutant histones were grown in 200 mL 2×YT until OD_{600} 0.4-.0.6 and then supplemented with 0.800 mM IPTG, 5mM nicotinamide, 0.02% arabinose, and 2 mM noncanonical amino acid at 37ºC for 12 hours. The subsequent steps follow the same procedure as their wild-type counterparts. Purified proteins were lyophilized and stored at -80ºC.

The general yield of the histone mutants varied greatly between 2 – 10 mg/L depending on the compatibility of the noncanonical amino acid with the PylRS mutant used.

SUMO-TEV-H4 Expression

His-tagged SUMO-TEV-H4 was grown in 1L 2×YT until OD_{600} 0.4-0.6 and then supplemented with 0.800 mM IPTG at 37ºC for 2 hours. The subsequent steps are identical overall to the purification of the other wild-type histones with the following differences: (1) Cells were lysed by sonication under gentler conditions at 4ºC, and (2) SUMO-TEV-H4 was washed on the Ni Sepharose™ 6 Fast Flow column (GE Healthcare) for 15 column volumes. The purified protein was then quantified and stored at -80ºC.

The yield of His-tagged SUMO-TEV-H4 was around 10 mg/L. The purity of SUMO-TEV-H4 is shown in Figure III-2.

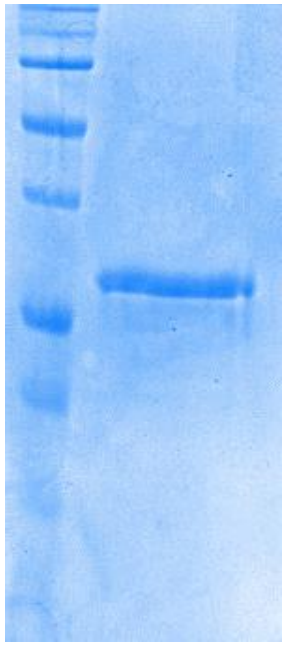

Figure III-2. 15% SDS Page gel depicting the purification of His-tagged SUMO-TEV-H4.

TEV Protease Expression and Purification

N-terminal His-tagged TEV protease was grown in 500 mL 2×YT media to OD_{600} 0.40–0.60 and then supplemented with 0.800 mM IPTG to induce expression for 4 hours at 37°C. Cells were then collected by centrifugation at 4 K for 20 minutes. Following resuspension with 30 mL of a lysis buffer containing 10 mM Na_3PO_4 (pH 8.0), 150 mM NaCl, 5 mM KCl, and 0.1% Tween 20, the cells were lysed by sonication at 4°C. The cell lysate was then centrifuged at 10 K for 40 minutes.

The supernatant was then collected and applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare) at 4°C. The column was washed with a chilled wash buffer containing 50 mM Na_3PO_4 (pH 8.0), 200 mM NaCl, 20 mM imidazole, and 10% glycerol for 10 column volumes. After wash, the enzyme was eluted with a chilled buffer containing 50 mM Na_3PO_4 (pH 8.0), 200 mM NaCl, 300 mM imidazole, and 10% glycerol. The enzyme was then dialyzed into a storage buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl, and 5 mM 2-mercaptoethanol at 4°C. Following dialysis, the protein concentration of the enzyme was determined using UV-Vis and then distributed into 5 μm aliquots and stored at -80°C until the time of use.

SIRT1 and SIRT2 Expression

HisTagged SIRT1 and SIRT2 were expressed using 2000 mL of 2YT media supplemented with 0.8 mM IPTG for 16 hours at 16°C. The cells were induced between OD_{600} 0.4-0.6. Cells were harvested and lysed by sonication in lysis buffer 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1mM phenylmethylsulfonyl fluoride, 0.1% Tween-20.

Following centrifugation, the supernatant was incubated with nickel-nitrilotriacetic acid resin for 1 hour at 4^{o}C. The resin was then loaded onto a column and rinsed with 100 mL wash buffer 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 20 mM imidazole, 5 mM 2-mercaptoethanol. Following wash, the protein was eluted with 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 300 mM imidazole, 5 mM 2-mercaptoethanol, concentrated, and loaded onto a Q Sepharose Fast Flow column (GE Healthcare). Fractions containing the enzyme were then collected, concentrated, and loaded onto a Superdex 200 10/300 GL column (GE Healthcare) for further purification (Figure III-3 & III-4). The final purified protein was dialyzed in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM 2-mercaptoethanol and stored at -80^{o}C in 10% glycerol at a final concentration of 5 μM.

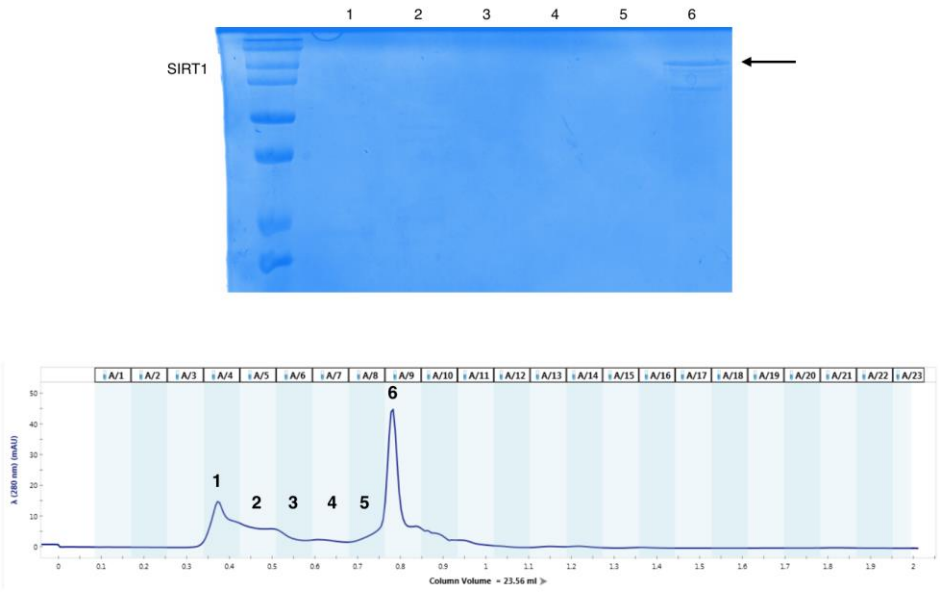

Figure III-3. SIRT1 purification on the Superdex 200 10/300 GL column.

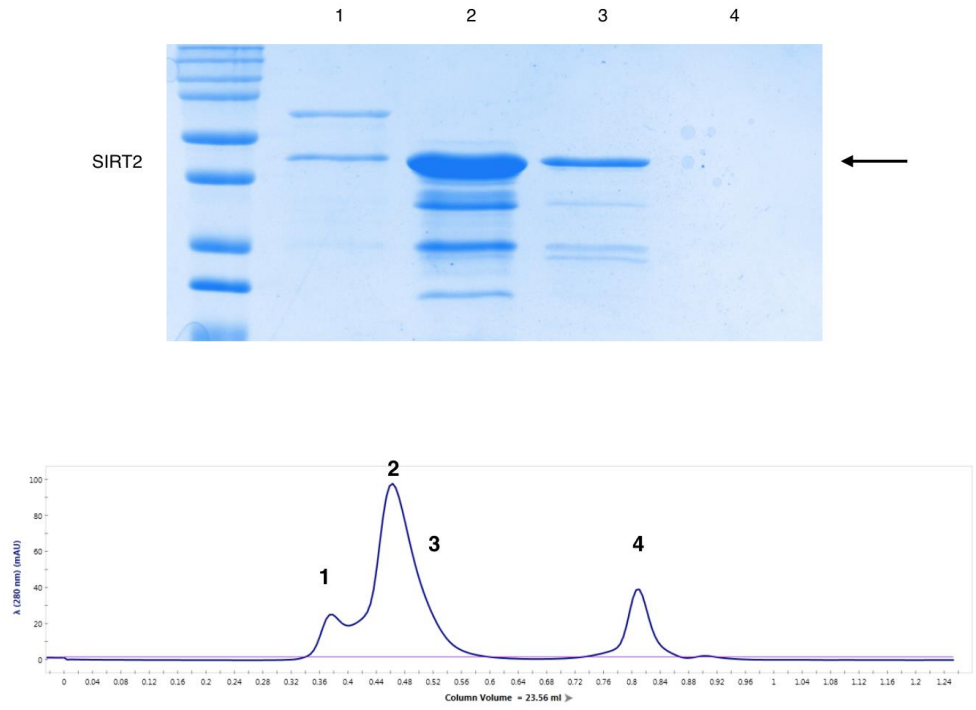

Figure III-4. SIRT1 purification on the Superdex 200 10/300 GL column.

Octamer Refolding

Histone octamers were assembled in the following manner. Recombinantly expressed HisTag-TEV-H2A, HisTag-TEV-H2B, HisTag-TEV-H3 (or HisTag-TEV-acetyl-H3), and HisTag-SUMO-TEV-H4 were dissolved in a histone unfolding buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 6 M urea in equimolar ratios. The mixture was dialyzed using Slide-A-Lyzer MINI Dialysis devices (3,500-Da cutoff) obtained from ThermoFisher Scientific at 4^{o}C against 4x 500 mL octamer refolding buffer containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl for 4 hours total. The concentration of octamer during refolding was 2.5 mg/mL. Following the completion of refolding, octamers were transferred to microcentrifuge tubes and spun down at 14,000 rpm for 1 minute to remove any precipitate. Following confirmation of octamer formation with SDS-PAGE, the histone octamer was incubated with TEV protease for 24 hours at 4^{o}C. The TEV protease treated octamer sample was administered to a Ni-NTA column to remove the digested HisTag, SUMO protein, the undigested octamer, and TEV protease. The remaining octamer was stored at 4^{o}C on ice for short-term and –20^{o}C for long-term storage. Typical octamer yields ranged between 60–80% (Figure III-5).

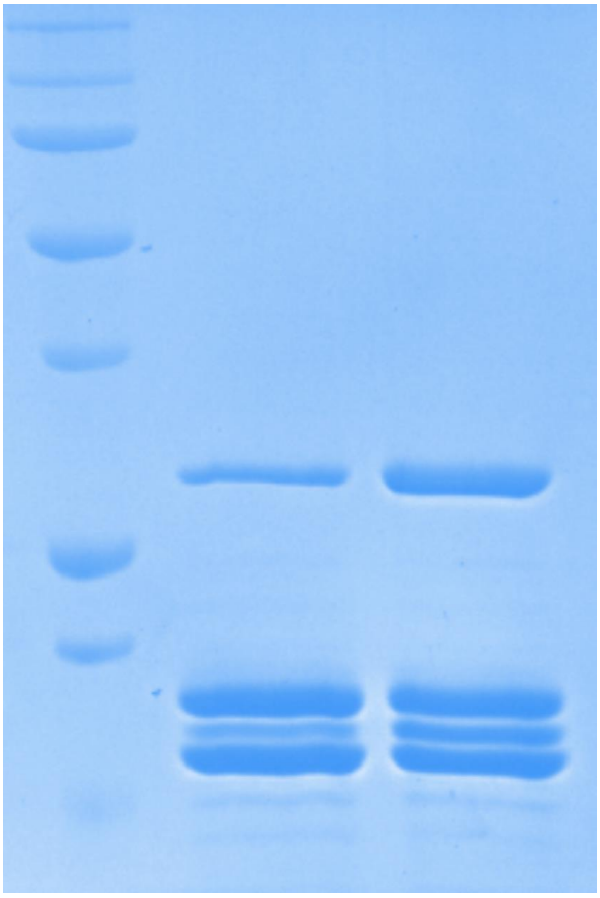

Figure III-5. Successful octamer refolding from two separate occasions.

P601 DNA Preparation

P601 DNA was prepared using a forward primer (5'-CTG GAG AAT CCC GGT GCC G-3') and a primer (5'-GAC AGG ATG TAT ATA TCT GAC ACG TGC C-3') from Integrated DNA Technologies to amplify the 147bp fragment P601 in the plasmid pGEM-3z-601 (Addgene) using PCR. For biotinylated-P601 DNA, a biotinylated reverse primer was used instead (biotin-5'-GAC AGG ATG TAT ATA TCT GAC ACG TGC C-3'). The PCR product was concentrated to 25 µM using general DNA concentration protocols and then used for subsequent nucleosome reconstitutions (Figure III-6).

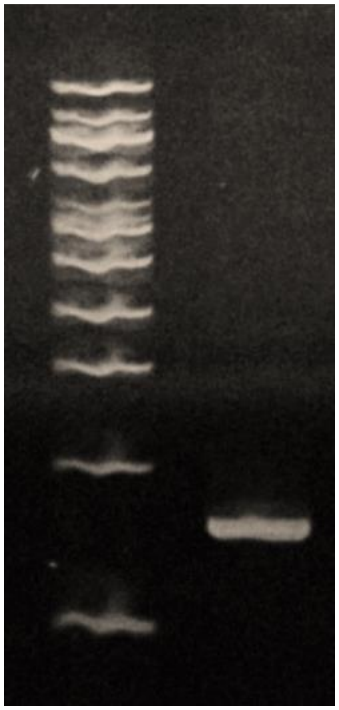

Figure III-6. PCR amplified P601 DNA fragment used for nucleosome reconstitution on 1% agarose EtBr gel.

Nucleosome Reconstitution

In a general reconstitution process, 2.8 µg (25 pmol) octamer was refolded with 2.2 µg DNA (25 pmol) using rapid salt gradient dilution starting from an initial salt concentration of 2 M NaCl to a final concentration of 250 mM NaCl in 30 minute time intervals with 10mM Tris (pH 7.5) buffer. Intermediate NaCl concentrations were 1.5 M, 1 M, 500 mM, and 250 mM. Reconstituted nucleosomes were stored at 4oC on ice for short-term storage until used. Nucleosomes were analyzed using 6% polyacrylamide-TBE native gels. Successful nucleosome reconstitutions were characterized by a band present between 400-500 bp DNA marker. Typical yields ranged between 60–100% (Figure III-7).

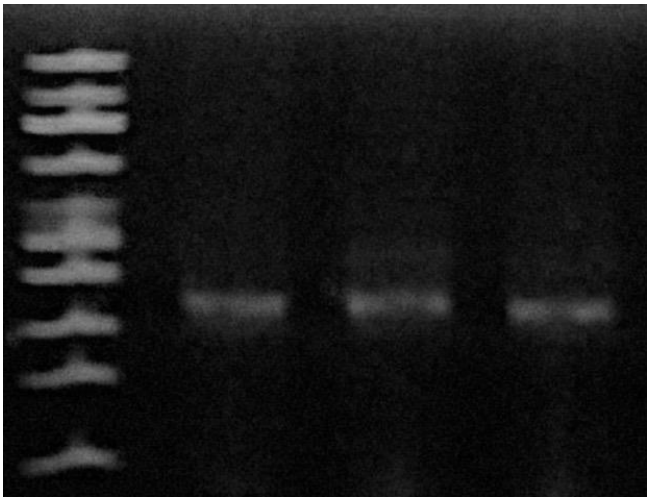

Figure III-7. 6% polyacrylamide-TBE native gel of various successful nucleosome reconstitutions. Nucleosome formation characterized by the appearance of a band between 400 - 500 bp.

Nucleosome Deacetylation Assays

Acetyl-nucleosome was bound to a streptavidin-coated 96-well plate with a buffer containing 20 mM Tris (pH 7.5) and 250 mM NaCl. 5 μM SIRT1 or SIRT2 (100nM final concentration) and 1 mM NAD^+ were added into wells to initiate the deacetylation process. In addition, wild-type nucleosome was provided in an adjacent well to serve as a negative control. The well in which no sirtuin enzyme was provided over the course of the assay (the 0 hour time point) was used as a positive control. Reactions were stopped at different time intervals by decanting solution and washing with a buffer with 20 mM Tris (pH 7.5) and 250 mM NaCl. Following the completion of the assay, the plate was blocked with milk for one hour, washed three times with PBST, and then incubated with pan anti-acetyllysine antibody for one hour. The plate was then washed five times with PBST with agitation for at least two minutes between each wash step. Subsequently the second antibody was applied to each well for one hour. Following the same wash procedure, the acetylation levels were detected using SuperSignal ELISA Pico Chemiluminescent Substrate from Thermo Fisher Scientific with a BioTek Synergy H1 microplate reader. The following results were then collected and processed using appropriate software.

Peptide Deacetylation Assays

Biotinylated acetyl-H3 peptides were analyzed in the same manner as in the nucleosome deacetylation assay.

Solution Phase Deacetylation Assays

Acetyl-H3 tetramers were incubated with 5 μM of their respective sirtuin enzyme for four hours at room temperature in a buffer with 20mM Tris (pH 7.5) and 250mM NaCl. The reactions were quenched and the conventional western blot procedure was carried out for analysis.

Gene Sequences

HisTag and Cleavage Site DNA Sequence

CATCACCATCATCACCACAGCCAGGATCCGGAAAATCTGTACTTCCAG

Histone H2A DNA Sequence

TCTGGTCGTGGTAAACAAGGTGGTAAAGCACGTGCAAAGGCTAAGACTCGT
AGCAGCCGTGCCGGTCTGCAGTTTCCAGTGGGTCGCGTTCACCGTCTGCTGC
GTAAAGGCAACTATGCTGAACGTGTGGGTGCTGGTGCACCGGTTTACCTGGC
AGCTGTACTGGAATATCTGACCGCAGAGATTCTGGAGCTGGCAGGTAACGC
AGCTCGTGATAATAAGAAGACCCGCATCATCCCACGTCACCTGCAGCTGGCC
ATCCGCAACGATGAGGAACTGAACAAACTGCTGGGCAAAGTTACTATCGCT
CAAGGTGGCGTTCTGCCGAACATCCAGGCAGTTCTGCTGCCGAAGAAAACC
GAATCCCACCACAAAGCGAAAGGTAAGTGA

Histone H2B DNA Sequence

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GTTACCAAGGCTCAGAAGAAAGATGGTAAGAAACGCAAACGTTCTCGTAAA
GAAAGCTATTCTGTGTACGTGTATAAAGTTCTGAAACAAGTACATCCAGACA
CTGGCATTTCCAGCAAAGCGATGGGCATTATGAACAGCTTCGTTAACGATAT
CTTCGAACGTATCGCAGGCGAAGCGAGCCGTCTGGCTCACTATAACAAACGT
TCTACCATCACCTCTCGTGAAATTCAAACTGCAGTTCGTCTGCTGCTGCCAGG
TGAACTGGCTAAACACGCGGTTAGCGAAGGCACTAAAGCAGTTACCAAATA
CACTTCTTCCAAATGA

Histone H3 DNA Sequence

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AAACAGCTGGCAACCAAGGCAGCGCGTAAAAGCGCTCCAGCTACTGGCGGC
GTGAAGAAGCCGCACCGTTATCGCCCGGGTACTGTGGCTCTGCGTGAAATCC
GCCGCTACCAGAAAAGCACCGAACTGCTGATTCGCAAACTGCCATTTCAACG
TCTGGTTCGCGAAATTGCTCAGGATTTCAAAACCGACCTGCGCTTCCAGTCT
AGCGCTGTGATGGCACTGCAAGAGGCGTCTGAGGCATATCTGGTTGGCCTGT
TCGAAGATACCAACCTGTGCGCAATCCATGCAAAGCGTGTAACCATTATGCC
GAAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H4 DNA Sequence

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CACCGTAAAGTGCTGCGCGACAACATCCAGGGTATCACCAAACCAGCTATTC

GCCGTCTGGCACGTCGCGGTGGTGTGAAACGCATCAGCGGTCTGATCTATGA

AGAAACCCGTGGTGTTCTGAAAGTATTTCTGGAGAACGTTATCCGCGATGCG

GTGACCTACACCGAACACGCGAAACGTAAGACCGTTACTGCTATGGATGTTG

TGTACGCTCTGAAACGCCAGGGTCGTACTCTGTACGGTTTCGGTGGCTGA

HisTag-SUMO DNA Sequence

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCG

GCAGCCATATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCA

AGCCAGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATC

TTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATG

GAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGT

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GGATAACGATATTATTGAGGCTCACAGAGAACAGATTGGTGGT

Histone H3K4TAG DNA Sequence

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CTGGTTCGCGAAATTGCTCAGGATTTCAAAACCGACCTGCGCTTCCAGTCTA

GCGCTGTGATGGCACTGCAAGAGGCGTCTGAGGCATATCTGGTTGGCCTGTT

CGAAGATACCAACCTGTGCGCAATCCATGCAAAGCGTGTAACCATTATGCCG

AAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H3K9TAG DNA Sequence

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GCGCTGTGATGGCACTGCAAGAGGCGTCTGAGGCATATCTGGTTGGCCTGTT

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AAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H3K14TAG DNA Sequence

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AACAGCTGGCAACCAAGGCAGCGCGTAAAAGCGCTCCAGCTACTGGCGGCG

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AAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H3K18TAG DNA Sequence

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AGCAGCTGGCAACCAAGGCAGCGCGTAAAAGCGCTCCAGCTACTGGCGGCG

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CGAAGATACCAACCTGTGCGCAATCCATGCAAAGCGTGTAACCATTATGCCG

AAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H3K23TAG DNA Sequence

GCTCGCACCAAACAGACTGCTCGTAAGTCCACTGGCGGTAAAGCGCCGCGT

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TCTGGTTCGCGAAATTGCTCAGGATTTCAAAACCGACCTGCGCTTCCAGTCT

AGCGCTGTGATGGCACTGCAAGAGGCGTCTGAGGCATATCTGGTTGGCCTGT

TCGAAGATACCAACCTGTGCGCAATCCATGCAAAGCGTGTAACCATTATGCC

GAAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H3K27TAG DNA Sequence

GCTCGCACCAAACAGACTGCTCGTAAGTCCACTGGCGGTAAAGCGCCGCGT

AAACAGCTGGCAACCAAGGCAGCGCGTTAGAGCGCTCCAGCTACTGGCGGC

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Histone H3K36TAG DNA Sequence

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Histone H3K56TAG DNA Sequence

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Histone H3K79TAG DNA Sequence

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GCCGCTACCAGAAAAGCACCGAACTGCTGATTCGCAAACTGCCATTTCAACG

TCTGGTTCGCGAAATTGCTCAGGATTTCTAGACCGACCTGCGCTTCCAGTCTA

GCGCTGTGATGGCACTGCAAGAGGCGTCTGAGGCATATCTGGTTGGCCTGTT

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AAAGACATCCAACTGGCTCGTCGTATCCGTGGTGAGCGTGCGTGA

Histone H3K115TAG DNA Sequence

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P601 DNA Sequence

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MmAcKRS DNA Sequence

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GAGCAGCAGGACTGCAAGAGCGCTCAGGCACCACAAATACAGGAAGACCTG

CAAACGCTGCAGGGTTTCGGATGAGGATCTCAATAAGTTCCTCACAAAGGCA

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CTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTG

ATAAACCCTGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAA

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GGGATTTCTACCAACCTGTAA

Protein Sequences

HisTag and Cleavage Site

GSSHHHHHHSQDPENLYFQ

Histone H2A

SGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLA
AVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGKVTIAQGGV
LPNIQAVLLPKKTESHHKAKGK

Histone H2B

SEPAKSAPAPKKGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQVHPDT
GISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVRLLLPGELAK
HAVSEGTKAVTKYTSSK

Histone H3

ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRR
YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN
LAAIHAKRVTIMPKDIQLARRIRGERA

Histone H4

SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEET
RGVLKVFLENVIRDAVTYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG

HisTag-SUMO

MGSSHHHHHHSSGLVPRGSHMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSE

IFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDII

EAHREQIGG

Histone H3K4 Mutant

ARTXQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRR

YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN

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Histone H3K9 Mutant

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YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN

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Histone H3K14 Mutant

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LAAIHAKRVTIMPKDIQLARRIRGERA

Histone H3K18 Mutant

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YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN

LAAIHAKRVTIMPKDIQLARRIRGERA

Histone H3K23 Mutant

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YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN

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Histone H3K27 Mutant

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YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN

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Histone H3K36 Mutant

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Histone H3K56 Mutant

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LAAIHAKRVTIMPKDIQLARRIRGERA

Histone H3K79 Mutant

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LAAIHAKRVTIMPKDIQLARRIRGERA

Histone H3K115 Mutant

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YQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTN

LAAIHAXRVTIMPKDIQLARRIRGERA

Results and Discussion

Design of Experiments for the Rapid Examination of SIRT1 and SIRT2 Activities

The basis of our approach is to utilize DNA-labeled nucleosomes—in our case, biotin-conjugated DNA—on an appropriate affinity-labeled 96-well plate for the rapid and efficient time-dependent screening of any enzyme (Figure III-8A). Our technique combines the ease and familiarity of western blot for detection and the efficiency of a rapid, streamlined nucleosome screening approach that is achieved when using 96-well plates. Furthermore, by placing the affinity tag on DNA rather than the nucleosome, we minimize the physical interference between the plate and enzyme during the screening process. Our aim during the method's development was to establish a straightforward and convenient technique that allows for rapid *in vitro* enzymatic profiling. As a demonstration of our technique, we report the deacetylase ability of SIRT1 and SIRT2 on multiple mono-acetylated histone H3 nucleosomes in a time-dependent manner.

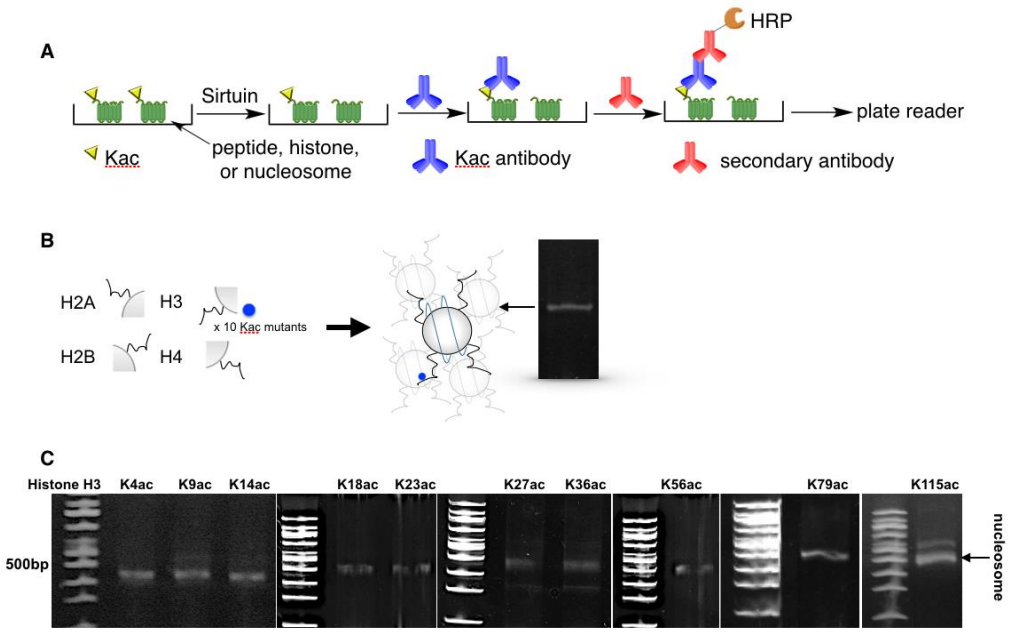


Figure III-8. An ELISA system for site recognition assays of sirtuin enzymes. (A) Peptides or nucleosomes with acetyllysine (Kac) were immobilized on the bottom of a 96-well plate. The deacetylation assay was then carried out with a sirtuin enzyme. After the reaction completed, they were probed by a pan anti-acetyllysine antibody followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. A chemiluminescent substrate was then provided and the signals were detected in a microplate reader. (B) Acetyl-nucleosomes were assembled by refolding equimolar amounts of histone H2A, H2B, H3, and H4 with 147bp P601 DNA. Nucleosome formation was determined using a gel shift assay. (C) 6%-TBE native gel of the ten unique acetyl-nucleosomes refolded in this study.

Expression of Acetyl-H3 Proteins and Reassembly of Acetyl-Nucleosomes

In order to profile lysine recognition sites of SIRT1 and SIRT2, histone H3 proteins with mono-acetylation at sites including K4, K9, K14, K18, K23, K27, K36, K56, K79, and K115 were expressed and refolded into nucleosomes together with biotin-conjugated P601 DNA and recombinant H2A, H2B, and H4 proteins (Figure III-8B–C). The expression of the acetylated histone H3 (acetyl-H3) proteins in *E. coli* was achieved by using a mutant acetyllysine-specific pyrrolysyl-tRNA synthetase together with its cognate $tRNA_{CUA}^{Pyl}$.

SIRT1 and SIRT2 Have Higher Activities on Nucleosomes than Peptides

As an initial study to demonstrate the viability of our method, we compared the enzymatic activities of both SIRT1 and SIRT2 on two separate substrate architectures—the peptide and the nucleosome. A notable increase was seen in the enzymatic activity for both sirtuin enzymes when incubated with a nucleosome assembled from histone H3K9ac (nucleosome-H3K9ac) against its acetylated peptide (peptide-H3K9ac) counterpart with SIRT1 and SIRT2 (Figure III-9). The rise in activity may be attributed to the possibility of stabilizing, secondary binding interactions that are achievable with the nucleosome substrate but not with the peptide, such as interactions with the N-tail or core region of another histone protein in the octamer. Therefore, acetyl-nucleosomes serve as more efficient and reliable substrates for both SIRT1 and SIRT2 over their peptide counterparts. For this reason, in the following experiments, only acetyl-nucleosomes were utilized for the assays of SIRT1 and SIRT2.

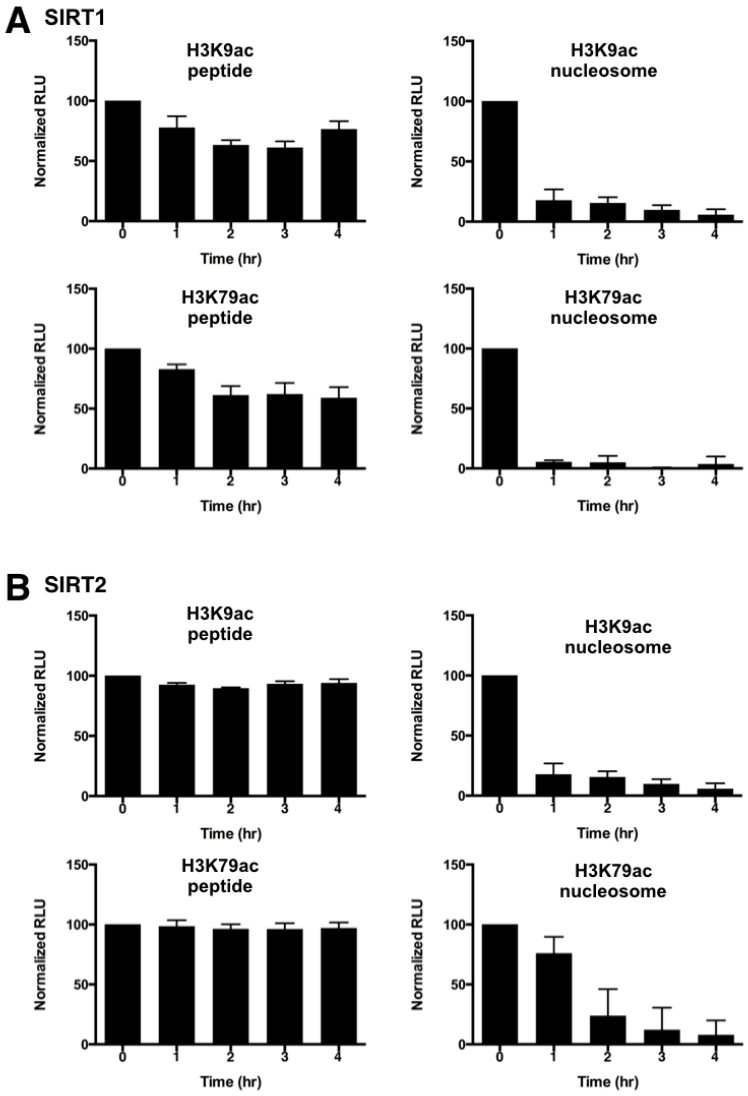


Figure III-9. Comparative study of SIRT1 and SIRT2 activity on acetyl-peptides and nucleosomes. (A) SIRT1 activity on peptides and nucleosomes containing an acetyllysine at histone H3K9 and H3K79. (B) SIRT2 activity on peptides and nucleosomes containing an acetyllysine at histone H3K9 and H3K79. Peptides and nucleosomes were immobilized by biotin on streptavidin plates.

SIRT1 Demonstrates Relatively No Selectivity on the Histone H3 N-terminal Tail

SIRT1 demonstrates relatively little substrate specificity amongst the pool of nucleosomes assembled from ten mono-acetylated histone H3 proteins (Figure III-10). Two exceptions in this study were histone H3K27ac and H3K56ac. We reason that due to the spacious nature of the SIRT1 active site, enzymatic deacetylation may be more dependent on the positioning of the acetyllysine toward the active site than the neighboring interactions between the adjacent residues on the substrate and the active site environment.[115]

When considering the catalytic residues involved in sirtuin-related deacetylation, most catalytically critical residues interact with the NAD^+ molecule rather than the substrate. The main documented interactions maintained between the sirtuin and acetylated substrate are through backbone interactions and surface contacts from residues four to five units away from the target modification rather than direct side-chain interactions in the active site; however, more distant residues may also influence deacetylation through other sirtuin interactions or through conformation-inducing interactions.[115, 116] Rauh *et al.* recently determined the specificity of SIRT1 across a wide acetylome peptide library.[92] In this study, they reported that SIRT1 displays some preferentiality with the neighboring residues surrounding the site of acetylation. SIRT1 exhibits a preference for mainly positively charged residues surrounding the acetylation site as well as non-charged residues at positions +2, +3, and –2, with a slight disfavoring for negatively charged residues surrounding the modification site. These features seemingly accommodate the binding groove of SIRT1 at which the peptides in the

microarray bound, which has a negatively charged bottom. One concern from the study; however, was that there is no guarantee that the native substrates would behave the same as their peptide counterparts due to the possibility of other auxiliary binding interactions unattainable through peptide studies. When applying the pattern determined by Rauh *et al.* to our acetylated nucleosome substrates, inconsistencies with SIRT1-targeted histone H3 lysine sites on the nucleosome profiled in the current study were observed. The surrounding sequences of the SIRT1-targeted histone H3 lysine sites demonstrated no convergence. Since the nucleosome is a robust and large complex, we reason that there must exist other stabilizing features that help overcome these perceived mismatches. In fact when considering the discoveries reported on acetylated histone H3 peptide studies, SIRT1 only demonstrates deacetylation reportedly at histone H3K9ac and to a lesser extent on H3K14ac.[92] However, based on the findings we report, SIRT1 demonstrates a wide range of deacetylase ability across the entirety of the histone H3 protein on the nucleosome.

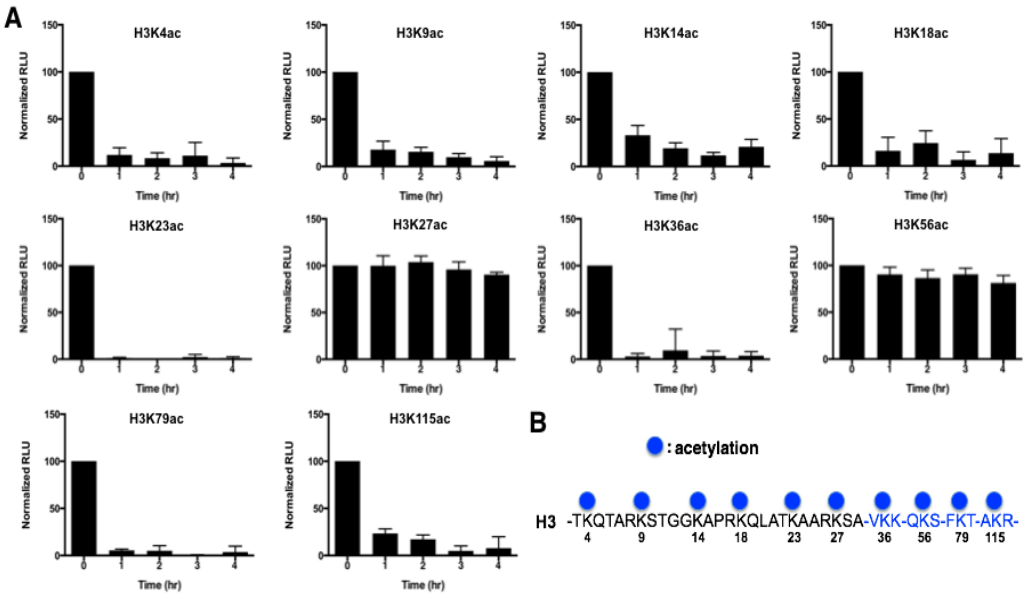


Figure III-10. The deacetylation activity of SIRT1 on ten acetyl-nucleosomes. (A) The activity of SIRT1 on ten selected histone H3 lysine sites. (B) The sequence of histone H3. The N-terminal tail region is displayed in black and the core region in blue.

When considering the criteria the enzyme must meet to initiate deacetylation, SIRT1 has two requirements: (1) the co-factor and substrate must both be bound and (2) the enzyme must enter its closed state.[115] In regards to these two requirements, it is plausible that the positioning of the acetyllysine is primarily mediated by the nucleosome complex, independent of its localization on the protein, meaning the adjacent histones—histone H2A, H2B, and H4—may interact with the sirtuin in a way as to aid the enzymatic reaction. Once positioned toward the active site, the SIRT1 enzyme can begin to exit its *apo* form and begin the deacetylation process.

As previous literature has reported, SIRT1 demonstrates the ability to deacetylate histone H3K9ac and H3K14ac, with a slightly higher preference for the former over the latter.[91, 114] Along with the remaining six sites that were deacetylated in our assay—histone H3K4ac, H3K18ac, H3K23ac, H3K36ac, H3K79ac, H3K115ac—, there was no significant outlier in relative enzymatic activity suggesting that SIRT1 activity is not predominantly determined by the adjacent amino acid sequence near the acetyllysine site but rather by some other undetermined enzyme-substrate binding factor.

Although nucleosome-H3K56ac was not deacetylated in our assay, several studies have reported its deacetylation *in vivo*.[117] We believe that since histone H3K56ac closely interacts with the nucleosomal DNA, the circumstance under which histone H3K56ac can be deacetylated may be dependent on the binding of other enzymes for stability or the loosening of DNA (or DNA damage) in the area near H3K56ac, features unattainable in our *in vitro* assay.[118]

In addition to nucleosome-H3K56ac, histone H3K27ac displayed no detectable deacetylation on the nucleosome. When considering the amino acid sequence near the site of acetylation, the region was mainly occupied by serine and alanine and an arginine directly adjacent to the modification site, residues with favorable binding interactions as described by Rauh *et al.*[92] In addition to the nucleosome study, we performed the deacetylation assay on the H3-H4 tetramer in the case that the DNA may have interrupted the deacetylation assay in some manner, which is discussed in further detail below. We hypothesize that similar to nucleosome-H3K56ac, nucleosome-H3K27ac has been made inaccessible to the sirtuin enzyme in some manner. From these studies, we conclude that SIRT1 does not recognize histone H3K27ac as a deacetylation target on the nucleosome *in vitro*; however, the ability of H3K27ac to be deacetylated *in vivo*, where the molecular system extends beyond simple nucleosome-sirtuin binding, remains to be seen.

SIRT2 Demonstrates Relatively No Selectivity on the Histone H3 N-terminal Tail

Akin to SIRT1, mammalian SIRT2 demonstrates essentially no substrate preferentiality on the ten acetyl-nucleosomes (Figure III-11). One exception in this assay was nucleosome-H3K56ac where previous *in vivo* data demonstrated deacetylation at this site by SIRT2.[117] This event may be similar to that of SIRT1 where SIRT2 requires other binding partners to aid in the deacetylation of this particular acetyllysine site due to its close interaction with the nucleosomal DNA. This would be achieved by either slightly unraveling the DNA or by binding to the DNA and thereby stabilizing the

docking of SIRT2 onto the nucleosome, or a combination of both events occurring. Alternatively, SIRT2 may interact with the H3-H4 tetramer—a native substrate *in vivo* that is absent of DNA—for the catalytic removal of acetylation at histone H3K56ac.

Since SIRT1 and SIRT2 belong in the same HDAC class, many of the details reported previously are relevant here as well. As reported with the SIRT1 catalytic site, most of the major SIRT2 catalytic contacts are with the bound NAD^+ molecule, leaving only basic backbone electrostatic interactions with the substrate.[115, 116] Though the catalytic core of SIRT1 and SIRT2 share a strong sequence identity, the catalytic pockets are not identical.[115, 116] The same peptide acetylome microarray discussed earlier with SIRT1 also analyzed SIRT2.[92] In this microarray, SIRT2 demonstrates a preference for a positively charged residue at the +4 position from the acetyllysine. In addition, the SIRT2 active site strongly disfavors negatively charged residues in the proximity of the modification site, which coincides with the active site's polar peptide-binding cleft and negatively charged bottom. In the same case as SIRT1; however, the sequence data appear to influence very little of the enzyme's specificity on the nucleosome. Previous studies on SIRT2 have only reported the enzyme's ability to deacetylate histone H3K9ac and H3K56ac.[91, 117] In this study, we have demonstrated that SIRT2 has the ability to deacetylate nine different histone H3 acetyl-nucleosomes. One exception to our study was the deacetylation of nucleosome-H3K56ac, which was addressed previously.

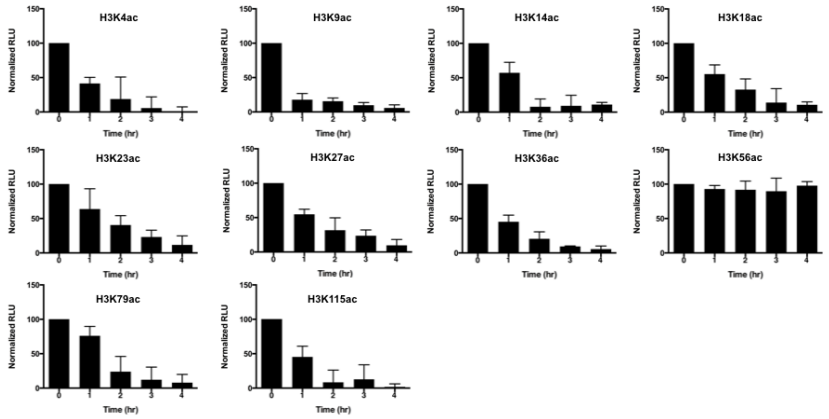


Figure III-11. The deacetylation activity of SIRT2 on ten acetyl-nucleosomes.

Like SIRT1, the catalytic core of SIRT2 can be categorized in two forms: open and closed.[116] In the *apo* state, SIRT2 exposes its hydrophobic core as well as the NAD^+- and substrate-binding sites. When both substrate and co-factor bind, the enzyme then enters its closed state.[115, 116, 119] From our SIRT2 deacetylation assay, no particular site where deacetylation was observed on the nucleosome architecture demonstrated a higher preferentiality over any other, suggesting that the presence of the nucleosome complex contributes a stabilizing force in SIRT2 activity.

SIRT1 Demonstrates the Ability to Deacetylate Tetramer-H3K27ac

As a follow up to our previous suppositions, we incubated tetramer-H3K27ac with SIRT1 in the solution phase to demonstrate the ability of the enzyme to remove the deacetylation mark in the absence of nucleosomal DNA. Indeed, SIRT1 does demonstrate the ability to deacetylate tetramer-H3K27ac (Figure III-12A). This finding upholds our previous assumption that SIRT1 can recognize histone H3K27ac; however, based on our nucleosome-H3K27ac and tetramer-H3K27ac data, the site itself does not appear to be a SIRT1 favored deacetylation site *in vitro*.

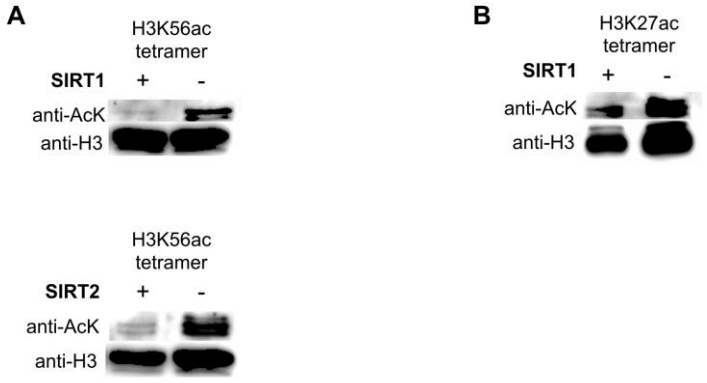


Figure III-12. Solution-based deacetylation assays of tetramer-H3K27ac with SIRT1 and tetramer-H3K56ac with SIRT1 and SIRT2. Experimental conditions were identical to the microplate-based assay. (A) Solution-based SIRT1 activity on tetramer-H3K27ac. (B) Solution-based SIRT1 and SIRT2 activity on tetramer-H356ac.

SIRT1 and SIRT2 Demonstrate the Ability to Deacetylate Tetramer-H3K56ac in the Solution Phase

In addition, we probed the ability of SIRT1 and SIRT2 to remove tetramer-H3K56ac (Figure III-12B). Both sirtuin enzymes demonstrated an apt ability to readily deacetylate the tetrameric protein in solution. We conclude in this study that the DNA in nucleosome-H3K56ac interferes with SIRT1 and SIRT2 binding; however, in the absence of the nucleosomal DNA, histone H3K56ac is readily removed by the two sirtuin enzymes.

Conclusion and Outlook

From the data presented in this study, we have demonstrated that both SIRT1 and SIRT2 show relatively little substrate specificity *in vitro*; however, several *in vivo* studies have presented evidence that suggests SIRT1 and SIRT2 have a higher preference for certain lysine sites over others.[91, 93] We believe that the substrate specificity of SIRT1 and SIRT2 *in vivo* may be dependent on their binding partners and the cellular context in which they are needed. For instance, following DNA replication the acetylation level at H3K56 rises when DNA damage accumulates during the replication process.[117, 120] In response, SIRT2 is co-localized in the regions of high histone H3K56ac levels with other DNA damage markers, such as p53, pATM, and CHK2, in order to repair the DNA damage.[118] Previous data have demonstrated a dependence on initial SIRT1-binding at regions of genomic instability for the recruitment of other enzymes. Dobbin *et al.* found that during the maintenance of genomic stability in neurons, HDAC1 recruitment to the DNA damage site cannot occur without SIRT1 preliminarily binding.[121] Other instances include the deacetylation of histone H3K9ac and H3K14ac as transcriptional repressing markers by SIRT1.[114] The deacetylation of histone H3K18ac by SIRT2 is seemingly situational as well, reported to function under the context of *Listeria monocytogenes* infection.[122] We believe our findings suggest a similar biological context in the examples above. Though SIRT1 and SIRT2 demonstrate the ability to remove acetylation from multiple sites along the histone H3 structure, the cellular context in which each deacetylation may occur remains to be discovered. Nevertheless, the implication of the two sirtuin enzymes and their non-

specificity implies a more flexible enzyme, able to respond to various cellular situations within the cell, rather than an enzyme specially designed for one singular task or function.

CHAPTER IV

ENZYMATIC PROFILING OF SIRT1 AND SIRT2 ON NINE CROTONYLATED AND BUTYRYLATED HISTONE H3 TETRAMERS

Introduction

Histone posttranslational modifications (PTMs) play a vital role in myriad cellular functions, such as gene transcription and repression, cellular health, chromatin compaction and decompaction, DNA repair, and more.[108, 109, 123-126] Crotonylation and butyrylation are newly discovered PTMs found on histones that potentially function as active gene promoters similar to their acetyl counterpart.[34, 36] A few studies conducted have discovered that histone acetyltransferases p300 and CBP can catalyze lysine butyrylation.[34, 36, 37, 127] In addition, histone deacetylase SIRT1-3 has been found to regulate histone H3K4cr levels *in vivo*, as well as decrotonylate histone H3K4cr peptides *in vitro*.[128] Despite the surging progress in the discovery of new histone acylations, there still exists a stunning lack of systematic studies done to elucidate how these new acylations interact with existing histone modifying enzymes.

Sirtuins are NAD^+-dependent histone deacetylases that have the ability to control gene expression. SIRT1 and SIRT2 are two of the seven enzymes in the sirtuin family localized in the nucleus.[128] A previous study conducted by the Liu lab has demonstrated that SIRT1 and SIRT2 display almost no substrate specificity across ten histone H3 lysine sites. We concluded in this study that SIRT1 and SIRT2 are flexible enzymes with the ability to respond to various cellular situations.[97] Since the discovery that

crotonyllysine can be a viable substrate for sirtuins, we sought to elucidate the role of SIRT1 and SIRT2 on other short-chain acyllysines.[128]

Here we implement our developed genetic noncanonical amino acid (ncAA) approach to express crotonylated and butyrylated histone H3 for the purpose of systematically studying the site and substrate specificity of SIRT1 and SIRT2 on the histone tetramer *in vitro*. From our study, we discovered that the two sirtuins demonstrate very little substrate specificity across the most of the histone H3 lysine sites. These findings support our previous supposition that SIRT1 and SIRT2 are enzymes of an adaptive nature with their specificity likely being tied to their binding partners.

Experimental Details

Cloning of Histone H3 Mutants

The human histone H3 gene was cloned into a pETDuet-1 plasmid. Site-specific amber mutations were made to the wild-type gene via Quikchange mutagenesis to obtain the desired mutagenetic feature (H3K4TAG, H3K9TAG, etc.) This process was repeated until all nine plasmids containing the desired histone H3 mutation were developed.

Expression and Purification of HisTag-SUMO-TEV-H4

A fusion gene coding an *N*-terminal His-tagged SUMO-linked to histone H4 with a TEV protease cleavage sit between them was cloned into a pETDuet-1 plasmid. The plasmid was then used to transform *E. coli* BL21 (DE3) cells. The transformed cells were then grown in 2×YT media to OD_{600} 0.4–0.6 and supplemented with 1 mM IPTG to induce expression for 4 hours at 37°C. Cells were then collect by centrifugation and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% NaN_3, and 0.1% Triton X-100. Cells were then lysed by sonication at 4°C on ice. The cell lysate was then centrifuged and the pellet was obtained, which was then dissolved in a lysis wash buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.1% NaN_3. The cells were spun down twice by centrifugation in the lysis wash buffer. Following the second wash, the cell pellet was then dissolved in a urea buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 6 M urea and cleared by centrifugation. The supernatant was then collected and applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare). The column was washed with a wash buffer containing 20 mM Tris-

HCl (pH 7.5), 500 mM NaCl, 20 mM imidazole, and 6 M urea. The protein was eluted with an elution buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 300 m M imidazole, and 6 M urea. Following purification, the proteins were lyophilized and stored at -80^{o}C.

Expression and Purification of Crotonyl- and Butyryl-H3 Proteins

To express crotonylated or butyrylated histone H3 proteins, pEVOL-MmBuKRS and a pETduet-1-based plasmid coding histone H3 with an amber mutation were used to transform *E. coli* BL21 (DE3) cells. Cells were grown in 2×YT media to OD_{600} 0.4–0.6 and the expression of the acylated histone H3 proteins were induced with an additional 1 mM crotonyllysine or butyryllysine, 5 mM nicotinamide, 0.2% arabinose and 1 mM IPTG for 12 hours at 37^{o}C. The subsequent purification was identical to the purification steps above. The purity of the proteins was visualized by SDS-PAGE (**Figure IV-1**).

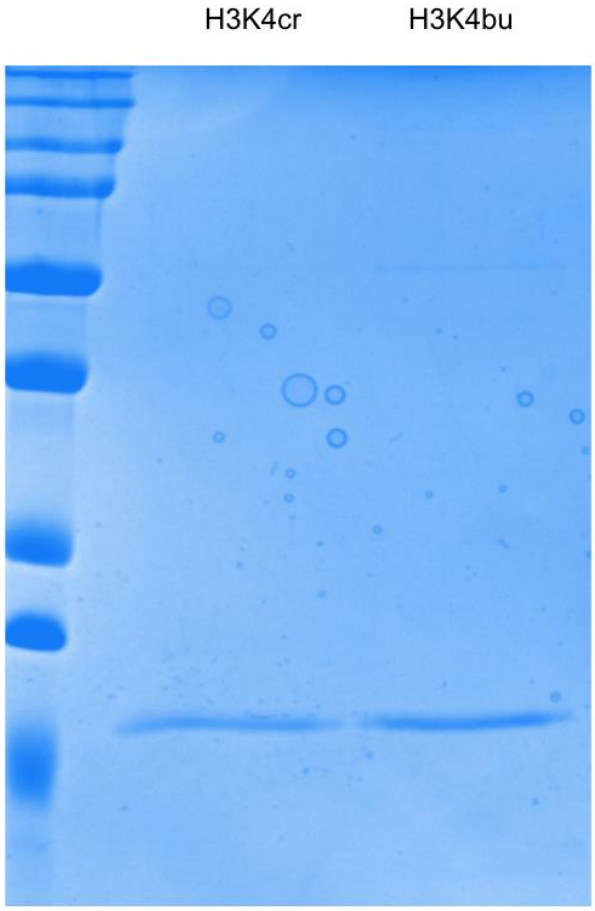


Figure IV-1. Expression of histone H3K4cr and H3K4bu.

Histone H3/H4 Tetramer Refolding

Histone H3/H4 tetramers were assembled in the following manner. Recombinantly expressed HisTag-TEV-crotonyl-H3 (or HisTag-TEV-butyryl-H3) and HisTag-SUMO-TEV-H4 were dissolved in a histone unfolding buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 6 M guanidinium chloride in equimolar ratios. The mixture was dialyzed using Slide-A-Lyzer MINI Dialysis devices (3,500-Da cutoff) obtained from Fisher Scientific at 4^{o}C against 4x 500 mL tetramer refolding buffer containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl for 2 hours total. The concentration of the tetramer during refolding was carefully adjusted to 2.0 mg/mL to avoid protein aggregation. After refolding, histone H3/H4 tetramers were transferred to microcentrifuge tubes and centrifuged at 14,000 rpm for 10 min to remove any precipitate. Following confirmation of tetramer formation with SDS-PAGE, tetramers were stored at 4^{o}C until use (**Figure IV-2B**).

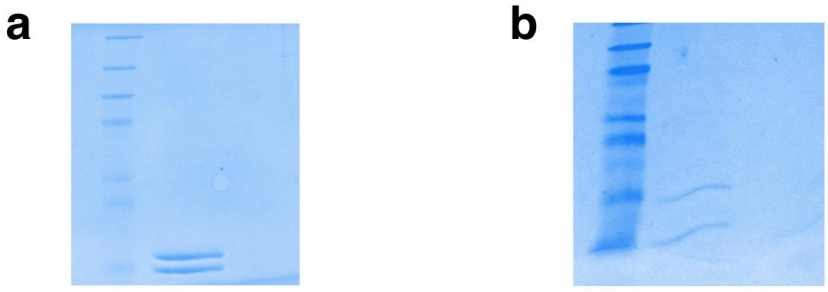


Figure IV-2. SDS-PAGE gel of A) histone H2A/H2B dimer and B) histone H3/H4 tetramer.

Expression and Purification of SIRT1 and SIRT2

N-terminal His-tagged SIRT1 and SIRT2 were expressed in 2000 mL of 2×YT media with 1 mM IPTG at OD_{600} 0.4-0.6 for 16 hr at 16^{o}C. Cells were harvested at 4000 rpm for 20 min and subsequently lysed by sonication in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Tween-20. Following centrifugation, the supernatant was incubated with Ni-NTA (nickel-nitrilotriacetic acid) resin for 30 min at 4^{o}C. The resin was then washed with 50 mL wash buffer 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 20 mM imidazole, 5 mM 2-mercaptoethanol. The protein was then eluted with an elution buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 300 mM imidazole, 5 mM 2-mercaptoethanol. The imidazole was then dialyzed out, and the sample was applied to a Q Sepharose Fast Flow column (GE Healthcare). The fractions containing the enzyme were collected and loaded onto a Superdex 200 10/300 GL column (GE Healthcare) for further purification. The final protein was dialyzed to a storage buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM 2-mercaptoethanol and stored at -80^{o}C in 20% glycerol at a concentration of 5 μM.

Tetramer Deacylation Assay

Crotonylated or butyrylated tetramers were incubated with 5 μM SIRT1 or SIRT2 and 1 mM NAD^{+} in a buffer containing 20 mM Tris (pH 7.5) and 500 mM NaCl for 2hr at 37^{o}C. Reactions were then quenched with 5mM nicotinamide and run on SDS-PAGE. Conventional western blot procedure was carried out for analysis using the

appropriate pan anti-acyllysine antibody (PTM Biolabs). An anti-H3 antibody was used as an internal control (Cell Signaling Technology).

Gene Sequences

Gene sequences for histone H3 (and its mutants), H4, HisTag, and HisTag-SUMO are available in Chapter III.

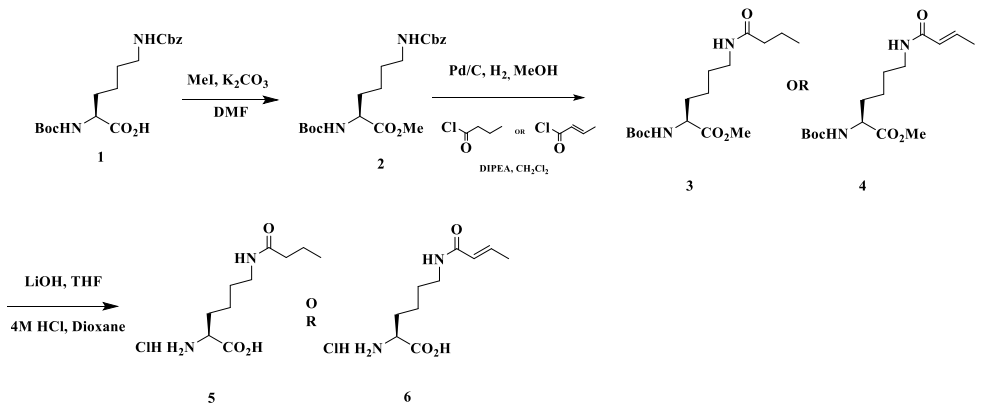
MmBuKRS DNA Sequence

ATGGATAAAAAACCACTAAACACTCTGATATCTGCAACCGGGCTCTGGATGT
CCAGGACCGGAACAATTCATAAAATAAAACACCACGAAGTCTCTCGAAGCA
AAATCTATATTGAAATGGCATGCGGAGACCACCTTGTTGTAAACAACTCCAG
GAGCAGCAGGACTGCAAGAGCGCTCAGGCACCACAAATACAGGAAGACCTG
CAAACGCTGCAGGGTTTCGGATGAGGATCTCAATAAGTTCCTCACAAAGGCA
AACGAAGACCAGACAAGCGTAAAAGTCAAGGTCGTTTCTGCCCCTACCAGA
ACGAAAAAGGCAATGCCAAAATCCGTTGCGAGAGCCCCGAAACCTCTTGAG
AATACAGAAGCGGCACAGGCTCAACCTTCTGGATCTAAATTTTCACCTGCGA
TACCGGTTTCCACCCAAGAGTCAGTTTCTGTCCCGGCATCTGTTTCAACATCA
ATATCAAGCATTTCTACAGGAGCAACTGCATCCGCACTGGTAAAAGGGAAT
ACGAACCCCATTACATCCATGTCTGCCCCTGTTCAGGCAAGTGCCCCCGCAC
TTACGAAGAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATG
AGATTTCCCTGAATTCCGGCAAGCCTTTCAGGGAGCTTGAGTCCGAATTGCT
CTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGGAAGAAAGGGAGAA

TTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGT
TTTCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGA
TGGGCATTGATAATGATACCGAACTTTCAAAACAGATCTTCAGGGTTGACAA
GAACTTCTGCCTGAGACCCATGCTTGCTCCAAACCTTTACAACTACCTGCGC
AAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCCCAT
GCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGC
TGAACTTCTGCCAGATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCA
TAATTACGGACTTCCTGAACCACCTGGGAATTGATTTCAAGATCGTAGGCGA
TTCCTGCATGGTCTGGGGGGATACCCTTGATGTAATGCACGGAGACCTGGAA
CTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTG
ATAAACCCTGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAA
ACACGACTTTAAAAATATCAAGAGAGCTGCAAGGTCCGAGTCTTACTATAAC
GGGATTTCTACCAACCTGTAA

Protein Sequence

Protein sequences for histone H3 (and its mutants), H4, HisTag, and HisTag-SUMO are available in Chapter III.



Scheme IV-1. Synthesis of N$^{\varepsilon}$-butyryl and N$^{\varepsilon}$-crotonyl-L-lysine hydrochloride.

Synthesis of N^{ε}-butyryl and N^{ε}-crotonyl-L-lysine Hydrochloride

Synthesis of Boc-Lys(Z)-OMe (2)

To a suspension of Boc-Lys(Z)-OH (**1**, 40.3 g, 0.106 mol) and potassium carbonate (27.6 g, 0.200 mol) in DMF (200 mL) iodomethane (9.90 mL, 0.159 mol) was added, and the mixture was stirred at room temperature for 30 h. The mixture was filtered, and the filter cake was washed with ethyl acetate (50 mL), dissolved in water (100 mL), and extracted with ethyl acetate (100 mL) two times. The ethyl acetate solutions were combined with the filtrate, and the solution was evaporated under vacuum until most of the DMF had been removed. The residue was redissolved in ether (250 mL), washed with water (100 mL) and brine (50 mL), dried (Na_2SO_4), and evaporated to afford **2** (41.8 g, quant.) as a yellow oil. The material was pure enough for the next-step reaction without further treatment.

Synthesis of N^{α}-Boc-N^{ε}-butyryl-L-lysine Methyl Ester (3)

A solution of **2** (4.20 g, 10.6 mmol) in methanol (100 mL) was hydrogenated under a H_2 balloon in the presence of palladium on carbon (10 wt.% Pd, 0.71 g, 0.67 mmol) at room temperature for 3 h, and TLC analysis showed a complete conversion. The mixture was then filtered over a pad of Celite and the solution was directly used for the next step reaction. The material was immediately used without purification since prolonged storage at room temperature or flash chromatography would contribute to lactam formation.

To a solution of the above amine (~9.15 mmol) in anhydrous dichloromethane (90 mL) cooled in an ice bath *N,N*-diisopropylethylamine (2.80 mL, 16.07 mmol) was added dropwise, followed by a solution of n-butyryl chloride (1.10 mL, 11.07 mmol) in dichloromethane (10 mL) dropwise over 20 min. The mixture was then stirred at room temperature for 12 h and was washed with sodium hydroxide solution (0.5 *N*, 20 mL) and brine (20 mL x2), dried (Na_2SO_4), evaporated, and flash chromatographed (EtOAc/hexanes, 1:3) to give **3** (2.77 g, 79% for two steps) as a colorless oil. R_f = 0.28 (EtOAc/hexanes, 1:2).

N^{ε}-Butyryl-L-lysine Hydrochloride (5)

To a solution of **3** (2.5 g, 7.57 mmol) in THF (30 mL) lithiumhydroxide solution (1.0 M, 50.0 mL, 15.0 mmol) was added, and the mixture was stirred at room temperature for 2 h. The mixture was diluted in water (20 mL) and extracted with ether (30 mL x 2). The ether extracts were discarded, and the remaining aqueous solution was adjusted to pH 3 with hydrochloric acid (3 *N*), with the concomitant formation of white precipitate. The suspension was extracted with ethyl acetate (60 mL x 2), and the combined organic phases were washed once with brine (30 mL), dried (Na_2SO_4), and evaporated to give the crude carboxylic acid as a colorless oil, which was used without further purification.

The above crude acid (~7.00 mmol) was dissolved in 1,4-dioxane (20 mL), and hydrogen chloride in 1,4-dioxane (4.0 M, 7.2 mL, 48.0 mmol) was added. The resulting white suspension was stirred at room temperature for 20 h, filtered, washed with

dichloromethane, and dried to give **5** (1.45 g, 76% for two steps) as a white solid. ^{1}H NMR (D_2O, 500 MHz) d 3.92 (t, 1H, J = 6.5 Hz), 3.04 (t, 2H, J = 6.5 Hz), 2.04 (t, 2H, J = 7.0 Hz), 1.84-1.75 (m, 2 H), 1.44-1.37 (m, 4 H), 1.38-1.25 (m, 2H), 0.72 (t, 3H, J = 7.0 Hz). ^{13}C NMR (D_2O, 125 MHz) 176.8, 171.7, 52.4, 38.6, 37.3, 29.1, 27.6, 21.4, 18.9, 12.5. The NMRs are shown in Figure IV-3 and IV-4.

Synthesis of N^{α}-Boc-N^{ε}-crotonoyl-L-lysine Methyl Ester (4)

Compound **2** (2.23 g, 5.65 mmol) was converted into the corresponding amine by hydrogenolysis, which was then treated with crotonyl chloride (0.81 g, 7.84 mmol) according to the procedure for **3** to give **4** (0.98 g, 53% for two steps) as a white oil. R_f= 0.35 (EtOAc/hexanes, 1:2).

N^{ε}-Crotonoyl-L-lysine Hydrochloride (5)

According to the same procedure for **4** (1.06 g, 2.84 mmol) **5** (0.597 g, 80% for two steps) was afforded as a white solid ^{1}H NMR (D_2O, 500 MHz) d 6.64-6.06 (m, 1H, 5.82 (dd, 1H, J = 1.5, 6.0 Hz), 3.92 (t, 1H, 6.0 Hz), 3.13 (t, 2H, 7.0 Hz), 1.87-1.80 (m, 2H), 1.78 (t, 3H, J = 2.0 Hz), 1.48-1.42 (m, 2H), 1.34-1.27 (m, 2H). ^{13}C NMR (D_2O, 125 Mhz) d 172.0, 168.8, 141.5, 123.8, 52.7, 38.6, 29.3, 27.8, 21.5, 17.1. The NMRs are shown in Figure IV-5 and Figure IV-6.

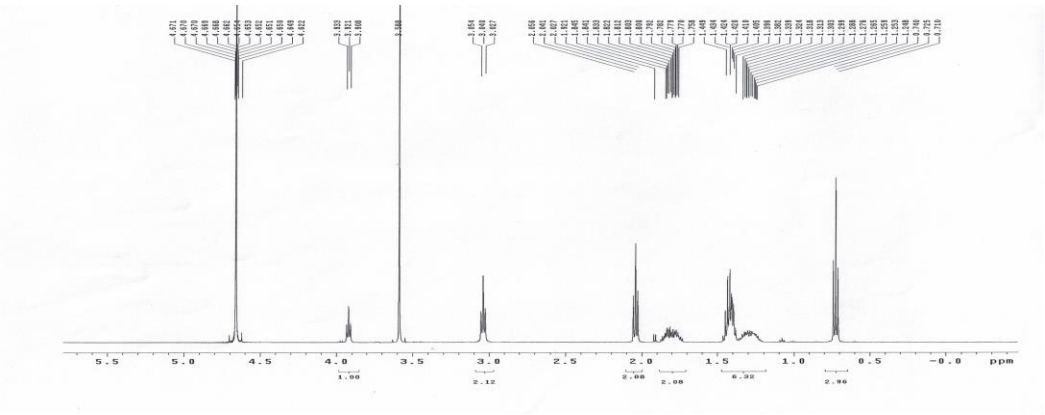


Figure IV-3. ^{1}H NMR spectrum for N^{ε}-butyryl-L-lysine hydrochloride.

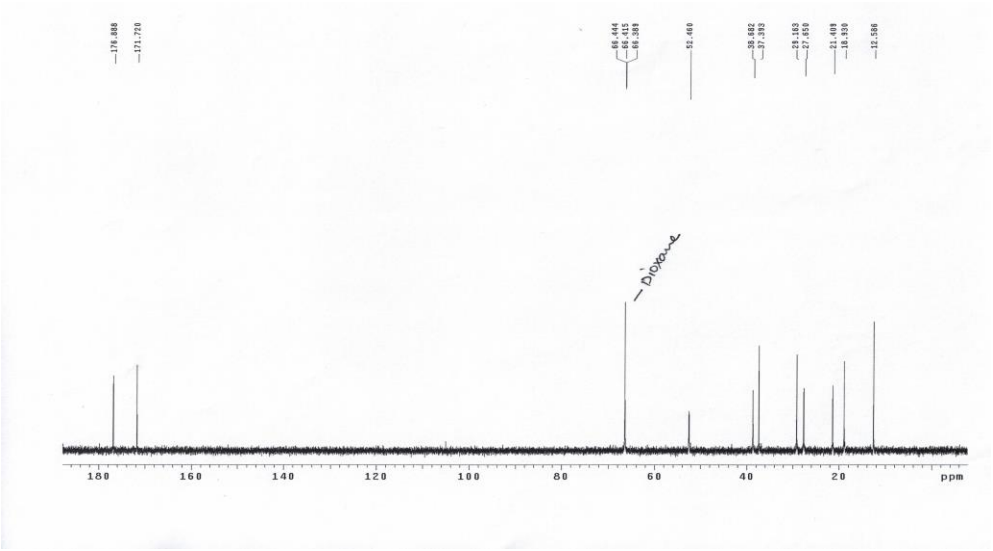


Figure IV-4. ^{13}C NMR spectrum for N^{ε}-butyryl-L-lysine hydrochloride.

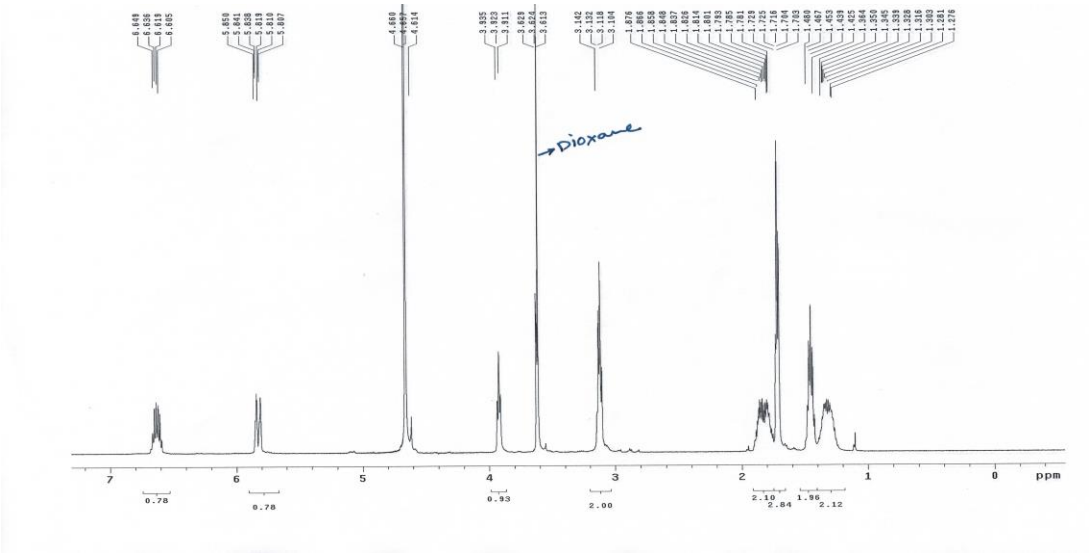


Figure IV-5. ^{1}H NMR spectrum for N^{ε}-crotonoyl-L-lysine hydrochloride.

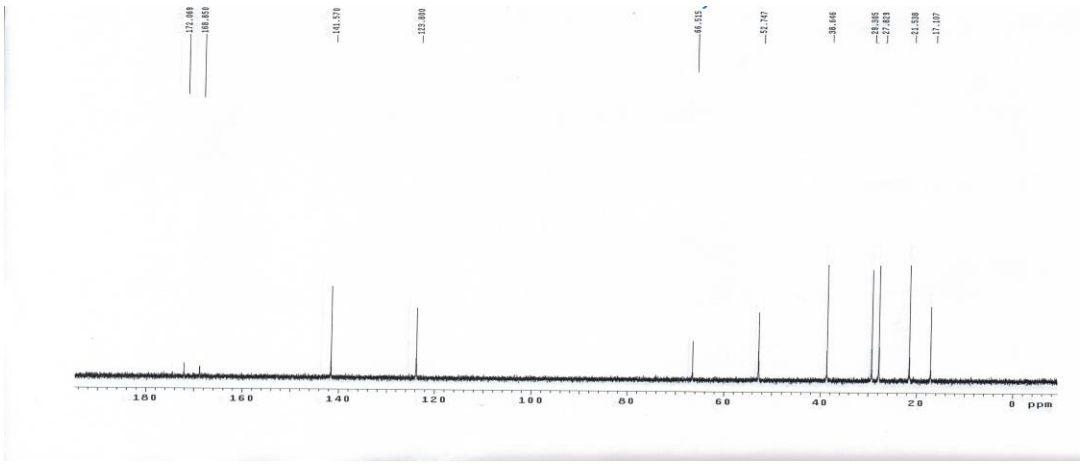


Figure IV-6. ^{13}C NMR spectrum for N^{ε}-crotonoyl-L-lysine hydrochloride.

Results and Discussion

Expression of Acyl-H3 Proteins and Reassembly of Acyl-Tetramer

Histone H3 proteins with monoacylation (either crotonylation or butyrylation) at sites including K4, K9, K14, K18, K23, K27, K56, and K79 were expressed and refolded into histone H3/H4 tetramer. The expression of crotonylated and butyrylated histone H3 proteins in *E. coli* was achieved by using a mutant pyrrolysyl-tRNA synthetase developed in our lab with its cognate $tRNA_{CUA}^{Pyl}$.[65]

SIRT1and SIRT2 Decrotonylation on Histone H3/H4 Tetramer

In our study, SIRT1 and SIRT2 demonstrated no substrate selectivity across the histone H3 *N*-terminal tail (Figure IV-7 & Table IV-1). As the enzyme reached the sites with more defined secondary structure (K36, K56, K79), the rate of deacylation slowed. This discovery largely coincides with our previous study on the deacetylase ability of SIRT1 and SIRT2 on acetylated nucleosomes.[97] In the study, we hypothesized the cause of the lack of specificity for these two enzymes was tied to their spacious active site. In addition, both SIRT1 and SIRT2 make basic electrostatic contacts on the protein backbone rather than on the acetyl moiety.[129, 130] For the decrotonylation of the crotonylated histone H3/H4 tetramer by SIRT1 and SIRT2, we apply the same logic; however, the results show a more logical trend this time around. The histone H3 lysine sites on the N-terminal tail (K4, K9, K14, K18, K23, K27) all demonstrate rapid decrotonylation when incubated with SIRT1 and SIRT2 at 37^{o}C for 2 hr. The core

histone H3 lysine residues (K36, K56, K79); however, show a decrease in the rate of decrotonylation. It is possible this trend is exhibited due to the *N*-terminal tail lacking defined secondary structure, whereas the core has a defined structure, creating a more stringent binding environment than the former.

SIRT1 and SIRT2 Debutyrylation on Histone H3/H4 Tetramer

Similar to our decrotonylation data, SIRT1 and SIRT2 demonstrate no substrate selectively on butyrylated histone H3/H4 tetramer. The same trend between *N*-terminal tail and core lysine residues is exhibited here as well (Figure IV-8 & Table IV-2). Due to the similar structure of crotonyllysine and butyryllysine, the expectation of similar results was partially expected. As mentioned earlier, since the major interactions of SIRT1 and SIRT2 with the substrate are on the protein backbone, the acyl modification likely does not affect the binding specificity. Therefore we conclude that the binding specificity is related to the structural conformation of the backbone at which the lysine site is located.

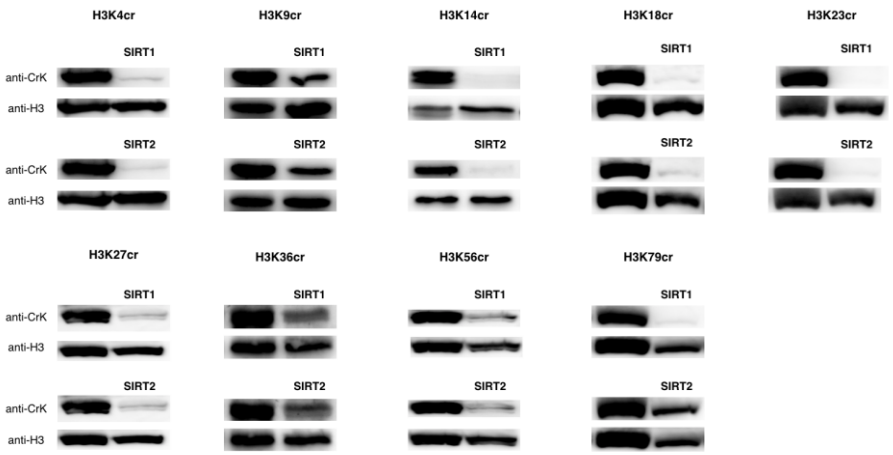


Figure IV-7. Western blot for SIRT1 and SIRT2 decrotonylation on histone H3/H4 tetramer.

Table IV-1. Histone H3 decrotonylation with SIRT1 and SIRT2. OOO: full deacylation; OOX: <70% deacylation; OXX: <20% deacylation.

	SIRT1	SIRT2
H3K4cr	**OOO**	**OOO**
H3K9cr	**OOX**	**OOX**
H3K14cr	**OOO**	**OOO**
H3K18cr	**OOO**	**OOO**
H3K23cr	**OOO**	**OOO**
H3K27cr	**OOO**	**OOO**
H3K36cr	**OOX**	**OOX**
H3K56cr	**OOX**	**OOX**
H3K79cr	**OOO**	**OXX**

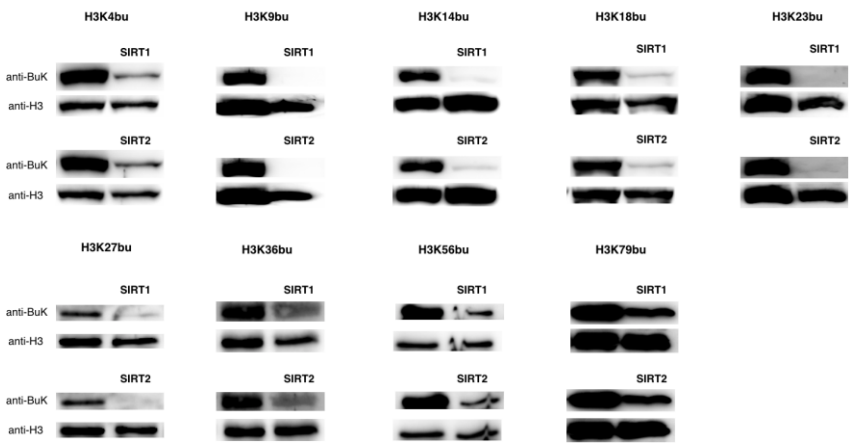


Figure IV-8. Western blot for SIRT1 and SIRT2 debutyrylation on histone H3/H4 tetramer.

Table IV-2. Histone H3 debutyrylation with SIRT1 and SIRT2. OOO: full deacylation; OOX: <70% deacylation; OXX: <20% deacylation.

	SIRT1	SIRT2
H3K4bu	**OOO**	**OOO**
H3K9bu	**OOO**	**OOO**
H3K14bu	**OOO**	**OOO**
H3K18bu	**OOO**	**OOO**
H3K23bu	**OOO**	**OOO**
H3K27bu	**OOO**	**OOO**
H3K36bu	**OOX**	**OOX**
H3K56bu	**OOX**	**OOX**
H3K79bu	**OOX**	**OOX**

Conclusion and Outlook

Protein lysine butyrylation and crotonylation are two fairly new acylations discovered by the Yingming Zhao group in 2007 and 2011, respectively.[34, 36] Beyond their discovery, there is a scarce amount of information related to the two acylations. From the data presented, we found that SIRT1 and SIRT2 demonstrate essentially no substrate selectively across the screened crotonylated and butyrylated histone H3 lysine sites. Previous evidence of sirtuin-catalyzed deacylation of crotonylation and butyrylation is limited to one study that found SIRT1-3 recognizes and deacylates a histone H3K4cr peptide.[128] Our study is significant in that similar to SIRT1 and SIRT2's lack of substrate specificity with acetylated histone H3 proteins, the enzyme's lack of substrate specificity on histone H3 is now expanded to include two new short-chain acylations: butyrylation and crotonylation.

Recent reports have discovered that the regulation of acetyl-CoA and crotonyl-CoA in cell are directly linked.[37] Typical cellular concentrations of crotonyl-CoA *in vivo* are generally low; however, when Sabari et al. purposely downregulated acetyl-CoA production, the concentration of crotonyl-CoA increased. This phenomenon suggests that acetyl-CoA and crotonyl-CoA may have competing roles in the posttranslational modification of their substrates. In complement with a study by Goudarzi and coworkers where they found that acetylation and butyrylation compete at histone H4K8, our previous and most recent study on SIRT1 and SIRT2 substrate specificity may suggest that acetylation, crotonylation, and butyrylation are competitors for histone H3 lysine

posttranslational modification.[35] From the study by the Yingming Zhao group, it is evident that these PTMs can modify many of the same histone H3 lysine sites.[34, 36] What determines which PTM is installed *in vivo* is most likely dependent on the cellular state or need rather than as a random occurrence. And the removal of these short-chain acylations can be regulated by the flexible and non-specific SIRT1 and SIRT2 enzymes.

CHAPTER V

CONCLUDING REMARKS AND FUTURE OUTLOOK

In this dissertation, we developed a method for nucleosome reconstitution using recombinantly expressed histone H2A, H2B, H3, and a SUMO-linked H4. We also demonstrated the genetic incorporation of a variety of noncanonical amino acids in histone using the evolved PylRS/$tRNA_{CUA}^{Pyl}$ pair.

Using the noncanonical amino acid approach, we developed a method for the rapid reconstitution and profiling of acetylated histone H3 nucleosomes on 96-well plates with SIRT1 and SIRT2. In addition, we expanded on the study by probing SIRT1 and SIRT2 activity on crotonylated and butyrylated histone H3/H4 tetramers. From the studies conducted, we discovered that SIRT1 and SIRT2 demonstrate almost no substrate specificity for the screened histone H3 lysine sites. Our discovery suggests that SIRT1 and SIRT2 may naturally have no substrate specificity; instead its binding partners, cellular localization, and cellular state determine the enzyme's specificity.

In order to further expand on our study of SIRT1 and SIRT2, we may begin to conduct more complex studies with SIRT1 and SIRT2 refolded with binding partners to see if any change in substrate specificity is seen. In addition, we can probe the substrate specificity of SIRT1 and SIRT2 on long-chain acylations, such as octanoylation and myristoylation. We can also study how different lengths of DNA wrapped around the nucleosome can affect SIRT1 and SIRT2 binding. Since the study of sirtuins is still in its

early ages of infancy, there remains much to be answered in relation to possible substrates, specificities, and the chemistry behind how sirtuins promote cellular health.

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